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PATHOLOGY OF AFLATOXICOSIS AND HEAVY METAL TOXICITY IN PEARL SPOT *ETROPLUS SURATENSIS* (BLOCH)

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

Fish and Fisheries Science (Mariculture)

OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
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AUGUST 2004

Dedicated to….
all my well wishers for their blessings and benevolence

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CERTIFICATE

Certified that the thesis entitled **"PATHOLOGY OF AFLATOXICOSIS AND HEAVY METAL TOXICITY IN PEARL SPOT, *ETROPLUS SURATENSIS* (BLOCH)"** is a record of independent bonafide research work carried out by **Mr. Anikuttan K. K** during the period of study from September 1999 to August 2004 under our supervision for the degree of **Doctor of Philosophy in Fish and Fisheries Science (Mariculture)** and the thesis has not previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title.

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ACKNOWLEDGEMENTS

At the outset, with deep sense of gratitude, I thank my major advisor, Dr.K.C.George, Principal Scientist, CMFRI, Cochin, for suggesting this topic to me. I also thank him for the invaluable advices and incessant encouragement and moral support, without which this thesis would not have seen the light of the day.

I am highly obliged to Prof (Dr) Mohan Joseph Modayil, Director, CMFRI, Cochin, for approving my research topic and also for the infrastructural facilities provided.

I place on record my indelible acknowledgements to my co-guide, Dr.K.S.Sobhana, Scientist (SS), CMFRI, Cochin, for the critical suggestions, timely help and encouragement throughout the period of my study at CMFRI.

I profoundly thank Dr.R.Paulraj, Principal Scientist and Head, PNP Division and PGPM, CMFRI, Cochin, for his scholastic advices and erudite suggestions after going through the manuscript.

I am greatly indebted to Sri. N.K. Sanil, Senior Scientist and In-charge, Electron Microscopy Laboratory, CMFRI, Cochin, for all the pains he has taken to help me for the ultrastructural studies besides critically going through the manuscript.

I thank my advisory committee members Dr. L.Krishnan, Principal Scientist, CMFRI, Cochin and Dr. T.V. Sathianandan, Senior Scientist, CMFRI, Cochin, for their invaluable advices and timely help.

My conscience won't forgive me if I don't acknowledge the help, co-operation, moral support and encouragement rendered by my juniors and friends, Neetha, Vinoj,

Radhika, Joice, Ramalinga, Ansy, Seema, Divya, Asha, Shiny, Annie, Sophia, Binu Gireesh, Aby, Patra, Chandu and Anand, I thank them all.

My special thanks are due to Unnikrishnan and Satish Sahayak for the cooperation, encouragement and timely help they provided.

I thank Sri.K.K.Surendran, Mrs..Vineetha, Sri. Palaniswamy, Sri. P.S. AnilKumar, Sri.Chandrasekharan, Mrs..Devaki,Sri.Paulton, Sri. M.D.Suresh, Sri.P.V.Joy and Sri.Jeevanraj, Staff of PNPD and PGPM for their co-operation and help.

I thank the entire staff members of Institute library and Hindi section for helping me in collection of secondary data related to my work and for the preparation of Hindi abstract.

I place on record my acknowledgements and gratitude to all my teachers whose blessings always helped me to surmount many a tussles in life.

Words cannot express my love, affection and indebtedness to my parents, brothers and sisters for their blessings, benevolence and encouragement without which I would not have reached where I am.

The financial assistance provided as senior research fellowship by the Central Institute of Fisheries Education (Deemed University, Indian Council of Agricultural Research, Versova, Mumbai) is greatly acknowledged.

31 August'2004 **(Anikuttan. K.K)**

Cochin

सारांश

पानी और आहार में मौजूद प्रदूषक और विषैले पदार्थों से जलजीवों में संघात होता है. आहार में दिखाए पडनेवाली विषैला वस्तु आफ्लाटॉक्सिन AFB1 और पानी में दिखाए पडनेवाला प्रदूषक काडमियम से मछली *इट्रोप्लस सुराटेन्सिस* (करिमीन) में होनेवाला रोगलक्षण इस अध्ययन का विषय है. अध्यनार्थ मछलियों को एक परीक्षण में 400 ppb के AFB1 में और दूसरे में 9.4 ppm काडमियमवाले पानी में 8 हफ्ते तक पालन किया. जन्तु के शरीरविज्ञानिय सूचकांक और जीवनागों में होनेवाला परिवर्तन का निरीक्षण किया. शरीरक्रिया में विषैला वस्तु (आफ्लाटॉक्सिन) के प्रभाव पर किए निरीक्षण ने व्यक्त किया कि लाल रक्ताणु (एरिथ्रोसैट) के काऊंट में पहले घटती और बाद में बढती होती है. श्वेतरक्ताणु (लूकोसैट), रक्त कोशिका राशि (PCV), सीरम अलकलैन फोसफेट, अस्पारटेट अमिनोट्रान्सामिनेस (AST) और अलनोन अमिनोट्रान्सामिनेस (ALT) में बढती हुई जबकि इसके विपरीत सीरम प्रोटीनों में घटती भी. महत्वपूर्ण अवयव जैसे जिगर, वृक्क,स्प्लीन और थैमस पर किए ऊतक रोगविज्ञानीय और परासंरचना अध्ययनों ने इन अवयवों के क्रमशः बिगड़ जाना दिखाई दिया. जिगर ऊतक विभाजन अध्ययनों ने कोशिकाओं के बलक्षय से अबुर्दकारी रोग लक्षण दिखाया. इस पर इलक्ट्रोन माइक्रोस्कोप पर किए निरीक्षणों ने कोशिकाओं के प्रत्येक सूक्ष्मभागों का अपचय सूचित किया. काडमियम से होनेवाले विषालू लक्षणों में ESR,AST और ALT में बढती देखी गई जबकि PCV, सीरम प्रोटीन्स और अलकलैन फोसफेट क्रियाकलाप में घटती दिखाई पडी. जिगर, वृक्क, स्पलीन, थैमस और क्लोम के कोशिका और उपकोशिका स्तरों में तीव्र अपचयन दिखाया पडा. जिगर में जमा हुआ ऊतकक्षय और अर्बुदकारी तंतुओं का बहुजनन व्यक्त था. वृक्कनाली कोशिकाओं का विशल्कन और ग्लोमुरले में पुंजन दिखाए पडे. क्लोम के अनुषंगी पटलिकाओं का लंबन और संलयन और एक अपलक्षण था. मुख्य अवयवों में दिखाए पडे अपचयों में ER विखंडन, माइटोकोन्ड्रिया का फुलाव, स्वतःकोशिका नाश (आटोफागिया), कोशभित्तियों और उन्हें जोडने के भाग माने डेसमोसोस का नाश परासंचरना अध्ययनों में मुख्य थे.

ABSTRACT

Food and water are the two major routes through which aquatic organisms are exposed to exogenous hazardous toxic insults. The pathology of the toxic impacts due to a food contaminant (aflatoxin B_1 or AFB_1) and an environmental pollutant (cadmium) was studied in pearl spot, *Etroplus suratensis* (Bloch). The influence of these two toxicants on various physiological indices as well as vital organs of the fish was studied in two separate experiments by exposing the fishes to either 400 ppb of AFB_1 or 9.4 ppm of cadmium for a period of 8 weeks. The physiological alterations due to aflatoxin exposure includes, an initial decrease followed by an increase in erythrocyte count; increase in leucocyte count, packed cell volume (PCV), serum alkaline phosphatase, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT); and a decrease in serum proteins. Histopathological and ultrastructural studies revealed progressive degenerative changes in the vital organs, liver, kidney, spleen and thymus. Liver sections revealed biliary proliferation and presence of pleomorphic, polyhedral and basophilic megalocytes invading into the normal parenchyma, which was diagnosed as hepatocellular carcinoma. The electronmicrographs revealed dilatation, fragmentation, proliferation and whirl formation of endoplasmic reticulum (ER), mitochondrial damages like condensation and loss of cristae and granules, nuclear changes like presence of perichromatin and chromatin granules, electron dense inclusions and presence of autophagosomes in the cytoplasm. The toxic insults due to cadmium were manifested as increase in ESR, AST and ALT and a decrease in PCV, serum proteins and alkaline phosphatase activity. Liver, kidney, spleen, thymus and gills exhibited severe degenerative changes at cellular and subcellular levels. Liver revealed coagulative necrosis and fibroblastic proliferation. The renal tubular epithelial cells were desquamated and periglomerular thickening as well as increased nuclearity were observed in glomeruli. The secondary lamellae of gills were elongated and fused together in cadmium exposed fishes. The major ultrastructural changes in the vital organs include ER fragmentation, mitochondrial swelling, presence of multivesicular bodies, autophagia, damage to desmosomes and cell membranes.

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Introduction

1. INTRODUCTION

The classical Malthusian theory of population proposed by Thomas Malthus in 1798 says that the human population will increase in a geometric progression where as food production will increase only in arithmetic progression. The essence of this theory is that, food production won't be sufficient to meet the requirements of the ever increasing population which is the major problem haunting the world today and food security is the most prioritized agenda among the policy decisions of all nations. Although there has been a lot of criticism over many of his views, the shortage of food due to population increase as visualized by Malthus, has stood the test of time. As population pressure started exhausting the land resources, man turned to water as an alternative to augment the food requirements and this paved the way for the emergence of aquaculture as a food production sector. Presently aquaculture ranks 4th in terms of global farmed meat production after pig meat, chicken meat and beef. The contribution of aquaculture to global supplies of fish, crustaceans and molluscs continues to grow, increasing from 3.9 percent of total production by weight in 1970 to 27.3 percent in 2000. Worldwide, the sector has increased at an average compounded rate of 9.2 percent per year since 1970, compared with only 1.4 percent for capture fisheries and 2.8 percent for terrestrial farmed meat production systems (FAO, 2002).

The developing countries are facing severe health problems due to lack of quality proteins in the diet of the masses. This can be met only by increasing the availability of good quality animal proteins. Since the cost of meat is very high, majority of the population cannot afford it. Hence, most developing countries have embarked on programmes, which produce affordable, good quality animal proteins. Fish offers the best alternative to high cost meat. More over, the high desirability and presence of polyunsaturated fatty acids makes it an ideal food supplement. In the coming years, aquaculture is going to be the thrust area for economic development in developing countries like India.

The major factors, which determine the success of an aquaculture operation are feed, environment and husbandry practices followed. Most of the feed ingredients used in aquaculture are of agricultural origin like oilcakes, cereals etc. These ingredients and the feed itself are very good substrates for the growth of toxigenic fungi which produce the mycotoxins. The most potent and widely reported mycotoxins are aflatoxins produced by the fungus of the genus *Aspergillus*. Tropical humid and hot climatic conditions favour infestation of the ingredients used in aquafeed manufacture by fungi. This leads to production of their metabolites and the feed get contaminated with aflatoxins. Thus aflatoxins reach the cultured fish/shellfish through the feed. Since the aflatoxins are highly carcinogenic and immunosuppressive (Manning, 2001; Sahoo and Mukherjee, 2001), it may become detrimental to the health of the animal.

Aflatoxicosis, the disease caused by aflatoxins has been widely studied in poultry and livestock compared to fish/shell fish. This may be attributed to the development of poultry and live stock industry earlier than aquaculture. The research on aflatoxins got an impetus with the outbreak of a disease of unknown etiology among young turkeys in poultry farms in Britain (Blount, 1961). More than one lakh birds died and the disease was called Turkey 'X' disease owing to the unknown etiology. A careful survey of the early outbreaks showed that they were all associated with feeds, mainly the Brazilian peanut meal (Blount, 1961). Further investigations of the suspected meal revealed the presence of a toxic factor in the feed, which was speculated to be of fungal origin. In 1961, the toxin producing fungus was identified as *Aspergillus flavus* and in 1962 a British interdepartmental working party on groundnut toxicity research gave the name aflatoxin to it by virtue of its origin. Near about the same time, during 1957-1960, epizootics of hepatoma were reported from trout hatcheries in USA. Discussions disclosed the fact that during the preceding years, the feed of trout had changed from a wet mixture of liver, raw fish, horsemeat etc. to dry pelleted feed. Although liver was involved in both trout and turkey epizootics, there were no suggestions that the causal factor might be the same because peanut meal was not a component of trout rations, instead cotton seed meal was used. But later studies confirmed that cottonseed meal was also contaminated by aflatoxins (Sinnhuber and Wales, 1978). Outbreaks of fish aflatoxicosis have also been reported from Germany (Wunder and Korn, 1982), Mexico (Ruiz Perez *et al.*,

Etroplus suratensis or pearl spot as it is commonly called, is a promising species for aquaculture in the Indian conditions (Somanath *et al.*, 2000) owing to its good growth rate, euryhaline nature, acceptance of artificial feed and compatibility in polyculture systems. As it is highly relished by consumers, especially in state like Kerala, it commands a good price also. The fish is cultured on a small scale in Kerala in the traditional 'pokkali' fields (paddy fields) and in the brackish water fish farms run by the Department of Fisheries, Government of Kerala. The species constitutes a part of the traditional culture systems of the Khazan lands and saltpan areas of Goa (Anon, 1995).

Aflatoxins as well as environmental pollutants pose serious health risks to the cultured fish/shell fish since food and water are the two major routes through which exogenous toxicants find entry to aquatic organisms. In this context the present study was taken up to understand the effects of aflatoxin and cadmium in the fish, *Etroplus suratensis* wherein aflatoxin served as the food contaminant and cadmium as the environmental pollutant. The fish is an ideal choice for studies related to nutritional pathology and ecotoxicology owing to its aquaculture potential as well as its wide distribution in the inland water bodies of peninsular India.

Hence, the present study was undertaken with the following objectives:

1. To understand the pathology of aflatoxicosis and cadmium toxicity in *Etroplus suratensis*

2. To evaluate the changes in enzymes profile due to the toxicities and correlate it with histopathological changes in vital organs.

3. To evaluate molecular and organ level pathology due to aflatoxin and cadmium.

4. To study the extent of insult by aflatoxin and cadmium on organs involved in the production of immunocytes.

Review of literature

2. REVIEW OF LITERATURE

2.1. AFLATOXICOSIS

2.1.1. Aflatoxins

Aflatoxins are a group of extremely toxic metabolites produced by the common fungi of the genera *Aspergillus.* The type of aflatoxins produced is highly variable between the different strains of *Aspergillus.* Most isolates of *A. parasiticus* produce the four main B and G aflatoxins while only the B aflatoxins are produced by *A.flavus* (Deiner and Davis, 1969; Ogundero, 1987). Chemically aflatoxins are polycyclic unsaturated compounds with a coumarin molecule flanked on one side by a bisfuran moiety and on the other side by either a pentanone for B-series or a six membered lactone for G-series (Coulombe, 1991). Although more than 18 aflatoxins have been isolated, only four are well known and studied extensively. They are AFB_1, AFB_2, AFG_1 and AFG_2. Two other aflatoxins, M_1 and M_2 are metabolites of B_1 and B_2 and are labelled so because of their presence in the milk of animals previously exposed to B_1 and B_2 (Sharma and Salunkhe, 1991).

Aflatoxins occur in a wide variety of commodities. Commodities of importance in terms of aflatoxin contamination worldwide include barley, beans, cassava, cowpeas, millet, peas, sesame, soybeans, sweet potatoes and wheat (Newberne and Butler, 1969). In Indonesia, samples from dry salty fish contained AFB_1 at an average level of 5µg/kg (Shank *et al.*, 1972). In the US, peanuts, corn, cottonseed, grain sorghum, millet, almonds and other tree nuts and dried fruits analysed also contained aflatoxins (Stoloff, 1976). In a survey of Thailand foods, aflatoxin has been detected in 5 percent of the dried fish samples at an average concentration of 166 µg /kg (FAO, 1979). Kalaimani *et al.* (1998) have reported the presence of aflatoxin in imported and indigenous brands of shrimp feeds collected from shrimp farms in Andhra Pradesh, and the levels ranged from10 to 130 ppb. In

Calcutta rice, pulses and oilseeds sold in local markets contained AFB_1 in the range of 333-10416 ppb (Begum and Samajpati, 2000).

2.1.1.1. Biosynthesis of aflatoxin

In the biosynthetic pathway, the chain is initiated by acetyl coenzyme A and malonyl CoA is the source of additional carbon units (Money, 1976). For AFB_1 the initial intermediate is norsolinic acid, which is converted to averufin in a two-step process via the intermediate averantin (Bennett *et al.*, 1980). The next step involves the ring opening of averufin followed sequentially by dehydration, epoxidation and epoxide rearrangement to form versicolorin A, which is then converted to the last major intermediate, sterigmatocystin via an oxido reductase and then finally to AFB_1 (Applebaum and Marth,1981). The biosynthesis of other aflatoxins as well as their metabolic relationship is less clear. According to Heathcote *et al.* (1973), AFB_1 can be readily converted by the fungus to other aflatoxins of the B and G class. However, Dutton *et al.* (1985) have proposed independent pathways for AFB_2 and AFB_1 and they have also confirmed the synthesis of AFM_1 and AFM_2 from analogous B toxins via mono oxygenase enzymes.

2.1.1.2. Metabolic transformation of aflatoxin

Metabolic transformation of aflatoxins especially, AFB_1, has been studied extensively. According to Post (1983) aflatoxins are absorbed from the diet in the alimentary canal and are passed to different organs. Majority of the metabolic conversions of AFB_1, is catalysed by cytochromes *P*-450, which are a group of mixed function oxidases present in the liver and other tissues. The major hydroxylated metabolites of AFB_1, formed by cytochromes *P*-450 are aflatoxin M_1 (AFM_1), aflatoxin P_1(AFP_1), aflatoxin Q_1(AFQ_1) and aflatoxin B_{2a}(AFB_{2a}). Aflatoxicol M_1 and aflatoxicol H_1 are also formed in smaller quantities. Most of the metabolites are less toxic than AFB_1 except AFB_1-2, 3,epoxide (or the 8,9 epoxide by IUPAC nomenclature) which is thought to be responsible for alkylation of cellular nucleic acids and subsequent carcinogenic and mutagenic activity (Coulombe, 1991).

Aflatoxin M_1 or 4-hydroxy AFB_1 is the first metabolite of AFB_1 isolated (Allcroft and Carnaghan, 1963). In a 12-month feeding study of rainbow trout, Sinnhuber *et al.* (1974) have observed that the carcinogenic activity of AFM_1 is roughly 25% of AFB_1. Hendricks *et al.* (1980a) have also observed that AFM_1 is non tumorogenic after a carcinogenesis assay with trout embryos. Experiments in many in vivo and in vitro systems have shown that the potential carcinogenic hazard posed by this metabolite is less than that of AFB_1 (Coulombe, 1991).

Aflatoxin Q_1, the 3- hydroxy metabolite of AFB_1 accounts for 30 to 50% of the metabolites produced from AFB_1 by microsomes from monkey and human liver (Masri *et al.*, 1974; Buchi *et al.*, 1974; Yourtee *et al.*, 1987) and is approximately 100 fold less hepatocarcinogenic in rainbow trout. (Hendricks *et al.*, 1980a,b).

Aflatoxin B_{2a} or the 2-hydroxy AFB_1 has been shown to be non toxic in vivo (Wogan, 1973) and non-mutagenic (Wong and Hsieh, 1976). The lack of biological activity of this is likely due to the absence of 2, 3 double bond which is known to be a requirement for the activation to a species that binds to DNA.

Aflatoxin P_1 is a product of the oxidative metabolism of AFB_1 and has been first identified as a major urinary metabolite from monkeys dosed with AFB_1 (Dalezios *et al.*, 1971; Dalezios and Wogan, 1972).

Metabolism of AFB_1 by NADPH - dependent reductases results in the formation of aflatoxicol (AFL) and the formation of AFL from AFB_1 is reversible. It also acts as a storage reservoir of AFB_1 which may act to enhance the ultimate toxicity of AFB_1 (Patterson, 1973). Trout has post mitochondrial enzymes which can convert back AFB_1 metabolites to AFB_1 (Loveland *et al.*, 1977) and this explains the extreme sensitivity of rainbow trout to AFB_1 especially since the trout system forms no other unbound metabolites in major amounts. Aflatoxicol is nearly as carcinogenic as AFB_1

in rainbow trout (Scheonhard *et al.*, 1981). In elasmobranchs, aflatoxin-metabolism yields aflaoxicol as the major metabolite, which can be demonstrated in vitro in liver post mitochondrial supernatant preparations of clearnose skate and nurse shark (Bodine *et al.*, 1989).

Two other reduced pentanone aflatoxins metabolites are known to exist, aflatoxicol M_1 and aflatoxicol H_1. Aflatoxicol H_1 is the hydroxylated oxidative metabolite of AFL, but has also been reported to be produced from AFB_1 and AFQ_1 (Salhab and Hsieh, 1975; Salhab and Edwards, 1977). Aflatoxicol M_1 or 4-hydroxy aflatoxicol is an oxidative metabolite formed from aflatoxicol. It may also be produced from AFM_1 by cytosolic reductases (Salhab *et al.*, 1977; Loveland *et al.*, 1983).

The most reactive metabolite of AFB_1 reported to be responsible for the carcinogenic and mutagenic effects is AFB_1 -2,3 epoxide (or 8,9 epoxide). This intermediate binds covalently with cellular macromolecules like DNA, RNA and proteins. The oxidative conversion of aflatoxins to the epoxide requires an unsaturated 2,3 bond in the terminal furan ring as in AFB_1 where as aflatoxins lacking this feature, such as AFB_2 do not bind to DNA (Swenson *et al.*, 1973). This has been further confirmed by Lutz *et al.* (1980), Marien *et al.* (1987), Loveland *et al.* (1987) and Loveland *et al.* (1988) who demonstrated the formation of DNA adducts with other aflatoxins (AFL, AFM and AFL-M_1) which possessed the unsaturated 2,3 double bond. Nunez *et al.*(1990), after experimenting with rainbow trout fry, have also found that the cytotoxicity and carcinogenicity depends on conversion of aflatoxins to electrophilic 8,9 epoxide.

AFB_1 - oxide can be inactivated by enzymatic conjugation with glutathione (Degen and Newmann, 1978). Such conjugation has been shown to be an important inactivation pathway in several species and the formation of AFB_1 – glutathione conjugates has been shown to protect against the hepatocarcinogenic effects of AFB_1 (Degen and Newmann, 1981; Lotlikar *et al.*, 1984).

2.1.2. Aflatoxicosis

Aflatoxin may be regarded as a quadruple threat - as a potent toxin, a mutagen, a teratogen and a carcinogen (Ueno and Ueno, 1978). The lethal toxicity of AFB_1 however, varies in different animals, from extremely susceptible (rabbit, duckling,trout, sheep, dog, rat) to resistant species (monkey, chicken, mouse).

Krishnamachari *et al.* (1975) have reported aflatoxicosis in humans from India. There are no toxicity values for humans but there is ample epidemiological evidence from case studies in Africa, South East Asia and India to implicate aflatoxins in the incidence of liver cancer and infant mortality (Hsieh, 1986).

Effects of feeds containing aflatoxins on livestock and poultry are well documented (Allcroft, 1969; Lovell, 1991a). Aflatoxicosis has been studied in numerous species including swine, cattle, goat, dog, chicken, turkey and other laboratory animals (Miller *et al.*, 1984).

2.1.2.1. Aflatoxicosis in fish and shellfish

The first attempts on fish- aflatoxicosis have been made by Ashley *et al.* (1964) and Halver (1965). Vosdingh and Neff (1974) have observed a dose of 0.02 µg/ml of aflatoxin B_1 to be sensitive on catfish cell lines and the rate of cellular degeneration was dose-dependent. Roberts and Sommerville (1982) have reported that in tilapia culture aflatoxicosis is a major cause of losses. When aflatoxin is fed at higher levels, it produces generalized haemorrhagic syndrome in fish (Poppe *et al.*, 1985). Liang *et al.* (1996) have noticed a reduction in survival rate of the prawn, *Penaeus chinensis* when the feed was contaminated with aflatoxin. According to Lovell (1991a), the sensitivity for mycotoxins varies among different species of fishes. Rainbow trouts are extremely sensitive to AFB_1 while other species, such as channel

catfish, are affected only at higher doses (Jantrarotai and Lovell, 1990; Jantrarotai *et al.*, 1990; Hendricks, 1994).

Channel catfish on a diet containing aflatoxin B_1 had high aflatoxin residues in flesh. However, when aflatoxin was removed from feed, the concentration of residues in flesh fell remarkably (Wu, 1999). Shrimps, particularly *Penaeus vannamei* consuming aflatoxin contaminated diets do not pass it to human consumers (Divakaran and Tacon, 2000).

Remedial measures to ameliorate the toxicity of aflatoxins have been a major area of interest to many researchers. Compounds like indol-3 carbinol, beta naphthoflavone and polychlorinated biphenyl complex - Aroclor 1254 could reduce the incidence of hepatocellular carcinoma in trout when they are fed these compounds prior to or after the exposure to AFB_1 (Bailey *et al.*, 1987). According to Winfree and Allred (1992), bentonite reduces the bioavailability of AFB_1, thus reducing the toxic effect. Chlorophyllin (CHL), a food-grade derivative of the ubiquitous green plant pigment chlorophyll, inhibit aflatoxin B_1 DNA adduction and hepatocarcinogenesis in the rainbow trout (Breinholt *et al.*, 1995). Takahashi *et al.* (1995) have reported the inhibition of aflatoxin- hepatocarcinogenesis by indol-3-carbinol but could not correlate it to the involvement of hepatic cytochrome *P*-4501A (CYP1A) induction. Two vitamin A2 compounds (3-dehydroretinol and 3-dehydroretinyl palmitate), were found to inhibit the AFB_1 induced carcinogenesis (Aboobaker *et al.*, 1997). The inhibition appeared to be due to the modulation of microsomal enzymes, which activate the carcinogen. Dashwood *et al.* (1998) have also found that chlorophyllin could inhibit AFB_1 - DNA adduct formation and hepatocarcinogenesis in trout. Liu *et al.* (2001) have reported the effectiveness of an intracellular enzyme, aflatoxin-detoxifizyme (ADTZ) from the edible fungus, *Armillariaella tabescens* for detoxification of aflatoxins.

Aflatoxicosis is an emerging fish health problem especially in the tropics owing to the immense scope and potential for the development of aquaculture as well as the favourable climatic conditions for fungal infestation of aquafeeds. It is recommended that more research be conducted on fungal toxins in fish feeds and that feed ingredients should always be tested for the presence of mycotoxins (Lovell, 1991b).

2.1.3. Effects Of Aflatoxins

Aflatoxins cause a variety of biological effects including hepatotoxicity, teratogenicity and immunosuppression (Pier, 1981).

2.1.3.1. Carcinogenesis

The carcinogenic potential of aflatoxins is well established and has been studied extensively in livestock and poultry. Among fishes, studies have been conducted in trout, salmon, channel catfish, tilapia and guppy (Halver, 1969; Lee *et al.*, 1971; Verma *et al.*, 1988; Jantrarotai *et al.*, 1990; Chavez *et al.*, 1994; Sato *et al.*, 1973). A common type of liver tumour seen in trout and salmon is hepatoma. These tumours appear in the form of epizootics in rainbow trout farms and hatcheries (Haddow and Blake, 1933; Nigrelli, 1954; Wales and Sinnhuber, 1966). Hepatomas have been induced for the first time in sockeye salmon by a diet containing 12 ppb aflatoxin B_1 by Wales and Sinnhuber (1972). The cause of high incidence of liver cancers in fish can be linked to aflatoxin contamination of feed (Wunder, 1974). Kumura *et al.* (1976) have stated that the occurrence of adenomatous polyps in the stomachs of hatchery grown trout and other species of fish is due to aflatoxin contamination of feed. These tumors develop mainly in the cardiac portion of the stomach, and are composed of hypertrophic, mucous-secreting cells, growing in papillary folds on stalks projecting into the gastric line. Wunder (1976) reported liver cancer in young rainbow trout due to feed contaminated by aflatoxins. According to Sinnhuber *et al.* (1977) feeding of aflatoxin contaminated feed can be linked to early reports of high incidence of hepatocarcinoma in rainbow trout. It is possible to produce liver cancer/hepatoma in rainbow trout by exposing fertile eggs and embryos to 0.5 to 1 ppm of AFB_1 (Wales *et al.*, 1978; Wales., 1979). Ruiz-Perez (1984) and Ruiz-Perez *et al.* (1984) have reported trabecular hepatomas in rainbow trout, with 2 ppb of aflatoxin in their livers. Majeed *et al.* (1984) have also observed high incidence of hepatoma in adult females of rainbow trout due to feed contaminated with aflatoxin. Rasmussen *et al.* (1986) have reported an outbreak of hepatocarcinoma in a Danish trout farm caused by aflatoxin-contaminated feed. Liver tumors can be

induced in rainbow trout (*Salmo gairdneri*) one year after injection of aflatoxin B_1 in the sac-fry stage of development. (Metcalfe *et al.*, 1988).

Although the carcinogenic effect of aflatoxin is more pronounced in livers of the animals, other organs may also be affected due to aflatoxicosis. In tilapia carcinogenicity is not only confined to liver, but also produce renal tubular carcinoma, lymphoma and hepatoma (Haller and Roberts, 1980). In coho salmon, aflatoxin does not produce hepatomas but liver lesions are seen which include necrosis of hepatocytes and fatty changes (Bruenger and Gruel, 1982). In addition to hepatoma, aflatoxin contamination of feed produces hepatic cirrhosis, cystic liver degeneration, cholangioma and hepatic adenocarcinoma in rainbow trout (Lopez-Jimnenez, 1983).

Bailey *et al.* (1994) studied the hepatocarcinogenicity of aflatoxin B_1 (AFB_1) and aflatoxicol (AFL) in rainbow trout. They have observed that both aflatoxins produce the same phenotypic response, predominantly mixed hepatocellular/cholangiocellular carcinoma. A three-fold greater carcinogenic potency for AFL is also observed. The relative carcinogenicities of four structurally related aflatoxins, aflatoxin B_1, aflatoxicol, aflatoxin M_1 and aflatoxicol M_1 in terms of their target organ DNA binding characteristics have been investigated by Bailey *et al.* (1998) in rainbow trout. They have found that, differences in tumorogenicity among the four dietary aflatoxins are largely or entirely accounted for by the differences in uptake and metabolism leading to DNA adduction, rather than any inherent differences in tumor initiating potency per DNA adduct.

Fibrosis and lymphocytosis are common in aflatoxin-induced hepatoma in rainbow trout (Wales and Sinnhuber, 1973). They have suggested that these reactions may control the growth of tumors and in some cases destroy them and the invasion of the tumors by lymphocytes is a host defense mechanism. They have also opined that the extent of fibrosis is age dependent and in advanced cases fibrosis appears to disorganize the tumor to a degree, which is suppressive. The lymphocytosis show a negative correlation with size and age of the hepatoma, being found almost exclusively in the very early' (small) hepatomas and preneoplastic nodules. Nunez *et al.* (1990) while studying the inter-relationships among AFB_1 metabolism, DNA-binding, cytotoxicity, and hepatocarcinogenesis in rainbow trout, have found that cytotoxicity, in common with carcinogenicity, is dependent on

metabolism of AFB_1 to the electrophilic 8,9-epoxide that can react covalently with cellular macromolecules, and that cytotoxicity contributes to, but is not required for, hepatocarcinogenesis. Nunez *et al.* (1991) have carried out ultrastructural investigations of AFB_1 induced hepatocellular neoplasms in rainbow trout. Large, usually uniform hepatic nuclei, large nucleoli, abundant, dilated rough-surfaced endoplasmic reticulum, and reduced glycogen storage are common findings in both hepatocellular adenomas and hepatocellular carcinomas. In addition, the presence of poorly developed microvilli in the space of Disse and in bile canaliculi, the occurrence of few or no bile preductule cells and a striking increase in the size and number of intercellular spaces characterize hepatocellular carcinomas.

The environmental temperature influences induction of tumours due to aflatoxin. Tumour incidences increase with high temperature. Rainbow trouts acclimated at low temperatures may show high DNA adduct formation but tumour induction is lesser (Zhang-Quan *et al.*, 1992., Curtis *et al.*, 1995).

There is considerable difference between species of fish in sensitivity to aflatoxins. Trout is more susceptible than salmon. In salmon AFB_1 produce benign hepatic adenomas where as in trout malignant adenocarcinomas are produced. There is also difference in AFB_1 metabolism, DNA adduct formation, adduct persistence and cytochrome *P*-450 isozyme composition in the livers of trout and salmon. AFB_1 - DNA binding is higher in trout than in salmon. AFB_1 - DNA binding favours cytochrome *P*-450 metabolism and subsequent formation of reactive 8,9-epoxide leading to tumour induction (Bailey *et al.*,1988; Nakatsuru *et al.*,1990). Among trouts itself, there exists differences in sensitivity between diploid and triploid individuals. Thorgaard *et al.* (1999) have reported a lesser incidence of aflatoxin induced tumours in triploid rainbow trouts than diploid fishes. They have suggested that triploids are more resistant to tumours because, it would be difficult to mutate or delete all three copies of tumour suppressor genes in triploids.

The genotoxic effects of aflatoxins have been established. However, in fishes there is a dearth of knowledge in this aspect of aflatoxicosis. Al-Sabti (1985) has studied the chromosomal aberrations in kidney cells induced by aflatoxins in three cyprinids, common carp, tench and grass carp. The aberrations increase in a dose dependent manner and also is species specific. AFB_1-DNA binding has been

studied in vivo in the medaka (*Oryzias latipes*) by Toledo *et al.* (1987). A linear dose response over the range 70-550 μgAFB_1 /kg body weight is noticed with maximum binding within the first 24 hr after intraperitoneal injection followed by a rapid loss of adducts. The effect of Aflatoxin B_1 on the chromosomal morphology of rohu and catla has been studied by Krishna and Gupta (2001). Intraperitoneal injection of sublethal doses of AFB_1 results in fragmented chromosomes, acentric chromosomes and ring chromosomes. The number of affected cells and chromosome spreads can be directly correlated with dose and exposure time.

2.1.3.2. Damage to vital organs

Besides carcinogenesis, aflatoxin brings about severe damages to many of the vital organs like kidney, liver, intestine, hepatopancreas, spleen etc. Aflatoxicosis results in the gross pathological changes like pale livers, general hemorrhage etc. Extremely pale livers have been observed by Jantrarotai *et al.* (1990) in channel catfish fed with diets containing aflatoxins at a dose of 12 mg/kg body weight. Pale livers and significant degenerative changes are observed in the internal organs of walleye after a 30 day feeding with aflatoxin incorporated feed (Hussain *et al.*, 1993). Aflatoxin at higher levels produces an acute toxic syndrome with massive focal necrosis, branchial oedema and a generalized hemorrhagic syndrome (Halver *et al.*, 1966; Halver *et al.*, 1969; Ashley, 1970). Bile duct hyperplasia is also considered as a diagnostic feature of aflatoxicosis (Halver, 1976).

Injection of 400 μg/kg doses of aflatoxin B_1 into *Salmo gairdneri* results in quantitative changes in the protein / DNA ratio of liver chromatin (Childs *et al.*, 1972). Carps are comparatively less sensitive to aflatoxins. Svobodova and Piskac, (1980) and Svobodova *et al.* (1982) have reported that, in carps aflatoxin does not produce any liver lesions. However, AFB_1 at higher doses such as 20 and 200g /kg feed result in histopathological alterations like dystrophy of liver tissue in carps (Svobodova *et al.*, 1981). Campos and Reyes (1985) have reported an acute liver necrosis in rainbow trout from fish farms in Chile where aflatoxins-contaminated fish meal is fed to fishes. Nearly 50% of the fishes were lost six months after initial

exposure. Tereza *et al.* (1987) have reported severe liver and intestinal damage in fishes from state farms of Portugal due to feed contamination with moulds and yeasts. Liver damage due to aflatoxins in rainbow trout fry is characterized by architectural disruption due to severe swelling of hepatocytes and necrosis (Nunez *et al.*, 1990). The viable remaining cells show pleomorphic atypical nuclei and foamy cytoplasm. Small basophilic cells emerge from degenerative hepatocytes. Also the cells show high mitotic index. Liver of channel catfish fed with aflatoxin contaminated feed have elicited marked variations from normal which includes necrotic foci with basophilic hepatocytes (Jantrarotai and Lovell, 1990). Spaces resulting from necrosis are present in the basophilic foci of liver. Jantrarotai (1991) has studied the effect of aflatoxin B_1 on channel catfish, *Ictalurus punctatus.* At subacute dose, necrosis and basophilia of hepatocytes are observed. Chavez *et al.* (1994) have observed severe damage to the liver of tilapia fed seven different levels of aflatoxins. In the liver, fatty infiltration of hepatocytes, nuclear and cellular hypertrophy, nuclear atrophy, increase in the number of nucleoli, cellular infiltration, cellular basophilia and necrosis were observed. The effect of AFB_1 on a cell line derived from the normal liver of a mature rainbow trout has been studied by Bechtel and Lee (1994). Alterations in the morphology and ultra structure and inhibition of DNA synthesis were observed. The effective concentration required for 50% inhibition (EC sub (50)) of DNA synthesis, after 2 days of treatment was 0.04 μ g/ml. Sahoo *et al.* (2001) have recorded necrotic and vascular changes in the liver of rohu (*Labeo rohita*) at acute doses of AFB_1 (7.50, 11.25 and 13.75 mg /kg body weight for ten days). During subchronic exposure (0, 1.25 and 2.50 mg/kg body weight for ninety days), preneoplastic lesions in liver were observed as a major histopathological alteration. Responses of Nile tilapia to varying concentrations of aflatoxin B_1 (AFB_1) have been investigated with diets containing 0, 0.25, 2.5, 10, and 100 mg AFB_1/kg of diet for 8 weeks by Anh Tuan *et al.* (2002). Livers of fish fed with diets containing 10 mg AFB_1 /kg contain excess lipofuscin and irregularly sized hepatocellular nuclei.

Aflatoxin also causes damages to other organs like kidney, intestine, spleen, heart, brain etc. Svobodova *et al.* (1981) have observed that AFB_1 at different doses of 2, 20 and 200 gm/kg of feed do not influence the weight, condition, health and physiological state of carp. However at higher doses, histopathological alterations can be seen characterized by circulation disorders in parenchymal organs

and gills, dystrophic change of nerve cells, and damage to kidneys. Aflatoxins produce chromosomal aberrations in the kidney cells of cyprinids within 48 hrs after injection (Al-Sabti, 1985). Verma and Pandey (1987) have observed dominance of lymphocytes containing pleomorphic nuclei in the kidney tissue of *Channa punctatus* due to the effect of aflatoxin B_1 which they refer as lymphosarcoma. Acute toxicity of AFB_1 in channel catfish, at a dose of 12 mg/kg body weight results in extremely pale gills, kidneys, spleens, stomachs, and intestines in moribund fish. Sloughing of intestinal mucosa and necrosis of hematopoietic tissues can be seen (Jantrarotai *et al.*, 1990). Kidney and digestive system have been affected in channel catfish fed aflatoxins contaminated feed (Jantrarotai and Lovell, 1990). Sinusoids of kidney are dilated and haematopoietic areas contain large number of immature erythrocytes. Intestinal mucosal epithelium accumulates excessive iron pigments. Necrosis of gastric glands in the stomach with infiltration of macrophages have been also observed. Jantrarotai (1991) has studied the effect of aflatoxin B_1 on channel catfish, *Ictalurus punctatus*. Damage to epithelial cells is prominent as indicated by severe loss of intestinal mucosa and necrosis of pancreatic acinar cells and gastric glands at acute doses of the toxin. At sub acute doses, enlargement of blood sinusoids in the head kidney, accumulation of iron pigments in the intestinal mucosal epithelium, and necrosis of gastric glands are observed. Chavez *et al.* (1994) studied aflatoxicosis in nile tilapia, *Tilapia nilotica.* Diets supplemented with 7 different levels of aflatoxin B_1 (0, 0.94, 1.88, 0.375, 0.752, 1.50, 3.0 mg/kg diet) were offered to 0.5 g nile tilapia for 25 days and subsequently maintained for 50 days on the basal diet without added aflatoxin. Fish samples from each treatment were taken on days 15, 26, 54 and 75 and preserved for histological examination. The changes in the kidney include congestion, shrinking of glomeruli and melanosis. The effects of AFB_1 at acute and sublethal doses on the various organs of rohu have been studied by Sahoo *et al.* (2001). At acute doses, necrotic and vascular changes in gill lamellae, meningitis and congestion in brain, degeneration and inflammatory reaction in heart along with degenerative to necrotic changes in kidney tubules and sloughing of the intestinal mucosa were observed. During subchronic exposure, changes in spleen, intestine, gill and pancreas were recorded. No lesions were observed in the spleen, stomach, pyloric intestine, head kidney or heart of Nile tilapia fed diets containing AFB_1 for 8 weeks. (Anh Tuan *et al.*, 2002).

Very few studies have been conducted on aflatoxicosis in shrimps. Aflatoxicosis in Pacific white shrimp, *Penaeus vannamei* has been studied by Ostrowski-Meissner *et al.* (1992) at different concentrations of the toxin in the feed. Hepatopancreas and the antennal gland are the two major organs affected. Juveniles of tiger prawn, *Penaeus monodon,* fed diets with different levels of aflatoxin B_1 (26.5 to 202.8 µg /kg) for 60 days have exhibited dose and time-related changes in tissues of the hepatopancreas, antennal glands and lymphoid organs (Lavilla-Pitogo *et al*, 1994). Atrophy of Restzellen (R-) cells, hemocytic infiltration and fibrosis in the intertubular sinuses of the hepatopancreas were recorded. Intertubular inflammatory responses were also seen in the antennal glands and lymphoid organs. Abnormal hepatopancreas and antennal gland tissues have been observed in *Penaeus vannamei* after exposure to AFB_1 at 50 ppb for two weeks by Ostrowski-Meissner (1995). Boonyaratpalin *et al.* (2001) have observed marked histological changes in the hepatopancreas of *Penaeus monodon* fed diet containing AFB_1 at a concentration of 100-2500 ppb for 8 weeks, which include atrophic changes, and necrosis of the tubular epithelial cells. Severe degeneration of hepatopancreatic tubules, infiltration of connective tissue into interstitial tissue of the hepatopancreas, as well as encapsulation of necrotic tissue/cells, were the other findings.

Few studies have been conducted on the disposition of ingested aflatoxins in the flesh or other organs of fishes. Liver and kidney are the main target organs accumulating the toxin. In carps there is no accumulation of aflatoxin in fish muscles (Svobodova and Piskac, 1980; Svobodova *et al.*, 1981; Svobodova *et al.*, 1982). Plakas *et al.* (1991), after studying the tissue disposition and excretion of AFB_1 have reported that there exists very low potential for the accumulation of AFB_1 and its metabolites in the edible flesh of channel catfish through the consumption of AFB_1 contaminated feed. Studies on the disposition of aflatoxin in rainbow trout have shown that there is a substantial absorption after oral administration, and that the highest tissue concentrations are reached in the liver (Ngethe *et al.*, 1992). However, between different species of fishes, variation in accumulation is observed, such as the hepatic accumulation of AFB_1 differ in the rainbow trout and tilapia (Ngethe *et al.*, 1993). The disposition of tritiated aflatoxin B_1 in the rainbow trout (*Oncorhynchus mykiss*) and the Nile tilapia (*Oreochromis niloticus*) following oral or intravascular administration has been studied by Horsberg *et al* (1994). High concentrations were

found in both the head kidney and trunk kidney and they suggested the possible toxic effects on the excretory and immunological functions of this organ.

2.1.3.3. Immunomodulation

In addition to the well-studied carcinogenic effects of AFB_1, these compounds can also suppress the immune system of fish (Manning, 2001; Sahoo and Mukherjee, 2001).

Pier *et al.* (1972) and Giambrone *et al.* (1978) have reported that AFB_1 affects the cell-mediated immune response, causing a reduction in the response of T-lymphocytes to phytohaemagglutinin, thymic involution and failure to develop immunity following vaccination in turkeys and in chickens. *In vitro* exposure of mouse splenocytes to AFB_1 results in a reduction of both B- and T-cell mitogenesis as well as a reduction in T-suppressor activity (Reddy *et al.*, 1987). Mice exposed to AFB_1 exhibit reduction in mitogen and mixed lymphocyte responses, primary antibody responses to T-dependent antigens, delayed type hypersensitivity reactions and cytotoxic T-cell function (Reddy *et al.*, 1987; Reddy and Sharma, 1989). Murine lymphocyte subpopulations show differential sensitivity to AFB_1 and T-cells are the most sensitive (Richard *et al.*, 1983; Reddy and Sharma, 1989). Weanling rats given oral doses of AFB_1 exhibit cell depletion in the bone marrow and thymus and a reduction of phagocytosis and macromolecular synthesis in macrophages (Raisuddin *et al.*, 1990). Chickens exposed to AFB_1 show reduced macrophage function (Michael *et al.*, 1973; Neldon-Ortiz & Qureshi, 1992) as well as suppressed cell-mediated immunity (Ghosh *et al.*, 1991). AflatoxinB_1 has been found to be a potent immunomodulator in endotherms. Exposure to AFB_1 results in the reduction of cytokine production in macrophages (Kurtz and Czuprynski, 1992) and T-cells (Marin *et al.*, 1996).

Similar to poultry and livestock, fishes are also prone to the immunosuppressive action of aflatoxins. Arkoosh and Kaattari (1987) have observed that embryonic exposure of rainbow trout to AFB_1 results in reduced B-cell memory.

Sensitivity of rainbow trout leucocytes to AFB_1 has been studied by Ottinger and Kaattari (1998) and in the aflatoxin exposed fishes, a decreased lymphocyte proliferation and immunoglobulin production in response to the mitogen, lipopolysaccharide were observed. These are thousand times more sensitive than murine leucocytes and seasonality in sensitivity was also recorded. During July – December period, the leucocytes were significantly more sensitive to AFB_1 than January-June period. Immunosuppressive effects of AFB_1 in *Labeo rohita* have been demonstrated by Sahoo and Mukherjee (1999). The aflatoxin-treated fish reveals reduction in total protein, globulin levels, bacterial agglutination titer, NBT, and serum bactericidal activities as well as enhanced albumin: globulin ratio without change in total protein concentration in comparison to control. Ottinger and Kaattari (2000) have exposed rainbow trout embryos to AFB_1 and suggests that exposure of embryo to AFB_1 results in long-term immune dysfunction. The effects of AFB_1 on non-specific immunity and disease resistance of rohu, *Labeo rohita,* have been studied by Sahoo and Mukherjee (2001). A single intraperitoneal injection of AFB_1 (1.25 mg/kg body weight) cause significant reduction in non-specific immunity as measured through serum bactericidal activity, lysozyme level, neutrophil oxidative activity, albumin:globulin ratio as well as reduced protection against *Aeromonas hydrophila* challenge when compared with control fish.

2.1.3.4. Biochemical alterations

Marked variations in biochemical parameters have been observed in cases of aflatoxicosis in terrestrial as well as aquatic animals. Chronic aflatoxicosis may be diagnosed by determining serum biochemical and haematological alterations before clinical symptoms become apparent (Kececi *et al.*, 1998). Determination of biochemical toxic effects of aflatoxins is important for diagnosis of aflatoxicosis in broilers (Rosa *et al.*, 2001). Aflatoxicosis in broilers may be manifested by decreased serum concentrations of total protein, albumin, total cholesterol (Kubena *et al.*, 1998; Oguz *et al.*, 2000), uric acid (Kececi *et al.*, 1998) and increased hepatic enzyme activities such as aspartate-amino-transferase and alanine-amino-trasferase (Amer *et al.*, 1998; Santurio *et al.*, 1999).

Liver enzymes like glucose-6-phosphate dehydrogenase, NADP-linked isocitrate dehydrogenase, lactate dehydrogenase and malate dehydrogenase have been studied in rainbow trout after feeding 20 ppb AFB_1 by Taylor *et al.* (1973). Except for a small increase in the activity of glucose-6-phosphate dehydrogenase, all other enzymes did not vary significantly. In channel catfish, AFB_1 at acute dose of 12 mg/kg body weight resulted in the lowering of haematocrits, hemoglobin concentrations, and erythrocyte counts to 10% of those in control fishes (Jantrarotai *et al.*, 1990). At sublethal levels, hematocrit, hemoglobin concentration, and erythrocyte count showed an inverse correlation with the dose of AFB_1 whereas leukocyte count increase with increasing toxin concentration (Jantrarotai and Lovell, 1990). Responses of channel catfish (*Ictalurus punctatus*) to subacutely and acutely toxic doses of AFB_1 have been studied by Jantrarotai (1991). The moribund fish following injection of acutely toxic doses of AFB_1 had rapid and severe anemia. Hematocrit, hemoglobin concentration and erythrocyte count were about 10% of those values in control fish. Subacutely toxic effects determined by feeding a semi-purified diet containing 0, 70, 330, 1,670, or 6,670 µg AFB_1/kg for 10 weeks include, lowering of hematocrit, hemoglobin concentration and erythrocyte count. Sarcione and Black (1994) have detected alpha foetoprotein (AFP)-like immuno reactivity in the sera of adult rainbow trout, with histologically confirmed hepatocellular carcinoma. The elevated serum AFP levels resemble those found in humans with the same malignancy and therefore they are of the opinion that serum-AFP measurements might be useful to confirm the appearance of hepatocellular carcinoma in experimental fish carcinogen-assay systems. Saber (1995) have observed that, dietary AFB_1 in *Tilapia nilotica* cause a reduction in total serum protein, albumin, globulin and acetylcholine esterase activity, where as the activities of lactic dehydrogenase, alanine amino transferase and aspartase amino transferase are significantly increased. Boonyaratpalin *et al.* (2001) have studied aflatoxicosis in *Penaeus monodon* by feeding the shrimps with diets containing different levels of AFB_1 for a period of 8 weeks. Total haemocyte count and phenoloxidase activity increase by the fourth week in shrimp receiving the highest concentration of AFB_1 and gradually it declined from the sixth to eighth week. Alkaline phosphatase and cholesterol in serum showed a high correlation with aflatoxin after 6 weeks of feeding where as calcium and phosphorus levels in the blood did not elicit any correlation with the aflatoxin concentration in diet. Anh Tuan *et al.* (2002) have exposed nile

tilapia to varying concentrations of AFB_1 (0, 0.25, 2.5, 10 and 100 mg AFB_1/kg of diet) for 8 weeks and observed a significant reduction in haematocrit at toxin concentrations above 0.25 mg/kg level.

2.2. CADMIUM TOXICITY

Cadmium as a pollutant gained worldwide attention with the outbreak of "itai-itai" disease in villages on the banks of the Jintsu river, Toyama Perfecture, Japan, in the year 1947. The name "itai-itai" (meaning "ouch-ouch") was so given in accordance with the patients' "shrieks" resulting from painful skeletal deformities (Kobayashi, 1971). It is estimated that approximately hundred deaths occurred until the end of 1965. The cause of the disease was traced to the consumption of cadmium contaminated rice from fields irrigated with water from Jintsu river which was polluted with the effluents from a nearby zinc mine. This gave an impetus to research on cadmium toxicity and at present, there is a plethora of information available on various aspects of cadmium toxicity.

The toxicity of cadmium is due in part to its competition with essential metals for binding sites and also its interference with sulfhydryl groups, which are essential for the normal functioning of enzymes and structural proteins. Cadmium blocks sulfhydryl groups in enzymes and competes for sites with zinc and calcium (WHO, 1971; Allen, 1994). Any enzyme with a thiol group is a potential target for these pollutants (Romeo, 1991). Cadmium is one of the most harmful heavy metals to animals and has a particularly long biological half-life (Biegniewska *et al.*, 1992) and the potential for exposure has increased with increasing industrial use of this metal (Park *et al.*, 1994). Toxicity of cadmium to fishes has stimulated considerable interest in recent years. (Sastry and Shukla, 1994). Results of the studies on toxic intracellular processes show that metals are transported through the biological membranes and interfere with biochemical functions. The stable state of cadmium in the natural environment is Cd^{2+}. Cd is an oxyphilic and sulphophilic element. It exists totally as the divalent species up to pH 8, in the absence of any precipitating anions such as phosphate or sulphide and begins to form $Cd(OH)^{+}$at pH 9 (Mohapatra and

Saha, 2000). Cadmium exposure has been established to induce cancer and circulatory diseases in laboratory animals and the International Agency for Research on Cancer has identified cadmium as a human carcinogen (Satoh-Masahiko *et al.*, 2002).

Cadmium sensitivity and bioaccumulation capacity are greater in marine invertebrates than in marine teleosts. This difference, which is explained in part by weaker diffusion barriers between seawater and internal organs, is correlated with a high density of calcium channels in the plasma membrane of muscular and nervous tissues of invertebrates (Daemers *et al.*, 1988). It was found that, the toxicity of cadmium was antagonized by low concentrations of manganese in the unicellular halotolerant alga *Dunaliella salina* (Rebhn and Ben-Amotz, 1988) suggesting a possible protection of primary phytoplankton producers by nutrient trace metals against toxic heavy metals present in the oceans.

The effect of salinity on the toxicity of cadmium to the bay mysid, *Mysidopsis bahia* Molenock, has been studied by De-Lisle and Roberts (1988). Mysids were more tolerant to Cd^{2+} at an intermediate salinity of 22 ppt and less tolerant at low and high salinity extremes. The toxicity also varies with the various inorganic salt forms in which it is exposed. Patel and Anthony (1991) have studied the 96-hr LC_{50} for cadmium in six species of tropical lamellibranchs. They are of the opinion that cadmium chloride has the highest LC_{50} value followed by nitrate, acetate, iodide and sulfate. Studies have shown that other metals, vitamins, chelating agents and protein diets which alter the physiological, biochemical and behavioral aspects in fish also influence cadmium toxicity (Sastry and Shukla, 1994).

The variation in the toxic nature of cadmium to different species of organisms is dependent upon a number of physiological characteristics. Species difference in sensitivity to cadmium toxicity exists between *Channa punctatus* and *Clarias batrachus* (Rana and Singh, 1996). Enzymological observations reveal that

C. punctatus is better equipped with conjugating enzymes than *C. batrachus* which makes it a more resistant species. Lyons *et al.* (1996), using immortalized cell lines have showed that mammalian cells are more sensitive to cadmium than fish cells. However, human epithelial explants are less sensitive to cadmium compared to rainbow trout epithelial tissue explants.

Living organisms have evolved several defensive mechanisms to overcome the Cd^{2+} toxicities and in eukaryotes, cells sequester Cd^{2+} as biologically inactive forms with cysteine rich peptides such as Glutathione (GSH), phytochelatins and/or metallothioneins (Mehra and Winge,1991; Perego and Howell,1997; Nies, 1999 ; Bruins *et al.*,2000; Hall, 2002; Cobbett and Goldsbrough, 2002).The mechanisms by which mammalian cells protect themselves against this toxic metal ion are very complex and not well understood.(Perego and Howell,1997; Zalups and Ahmad, 2003).

Some substances are found to reduce or nullify the toxic effects of cadmium. A membrance therapeutic drug 'Essentiale' (Natterman, FRD) was found to be effective in combating the cadmium induced structural and biochemical changes in the intestine of *O. mossambicus* (Kothari *et al.*, 1999). Another substance, zeolite is also found to reduce cadmium toxicity in *Heteropneustes fossilis* and *O.mossambicus* (James and Sampath, 1999; James, 2000). Quick lime (CaO) has the property to reduce cadmium toxicity (Kaviraj and Dutta, 2000). Shaffi *et al.* (2001) have studied the efficacy of selenium and zinc to combat the cadmium toxicity in *L rohita*. They are of the opinion that, selenium prevents cadmium-induced necrosis, blood pressure, injury to pancreatic beta cells and induction of hepatoglucogenic enzymes. Zinc also neutralizes the toxic effect of cadmium. The protective action of humic substances and calcium on cadmium toxicity has been studied in zebrafish (*Danio rerio*) by Meinelt *et al.* (2001).

2.2.1. Effects on vital organs

In case of mammals, the major organs and organ systems in which pathological changes are noticed as a result of cadmium toxicity include liver (Hogstrand and Haux,1990; Friedman and Gesek,1994), kidney (Hogstrand and Haux,1990; Novelli *et al.*, 1999) , brain and nervous system(Provias *et al.*, 1994), testes(Shen and Sangiah, 1995), spleen and bone marrow(Yamno *et al.*, 1998).

Almost all these organs are affected in fishes also. Giles (1988) opined that gills, liver, and kidney are the major organs affected. However studies have revealed the toxic impacts of cadmium in few more organs, viz, skin, digestive glands, olfactory system, gonads and heart. Lesions have been observed in the gills of the fish, *Lepomis macrochirus* on exposure to cadmium by Versteeg and Giesy (1986). Gill *et al.* (1988) have observed severe damage to the gills of the fish *Puntius conchonius* on exposure to sublethal levels of cadmium. The secondary gill lamellae showed disrupted epithelium, necrosis, accumulation of cellular debris, capillary congestion, and wilting of the pillar cell system. Hypertrophy and hyperplasia of chloride cells as well as partial or complete fusion of secondary lamellae were also seen. The gills of the mussel, *Mytilus edulis* on exposure to cadmium evokes an inflammatory reaction which begins with enlargement of the post lateral cells, followed by the dilation of blood spaces and migration of granular hemocytes to the epithelia (Sunila, 1988). In *Tilapia mossambica*, 50 ppm of $CdCl_2$ in rearing water caused lethal effects and 5 ppm sublethal effects. Engorgement of blood vessels, vacuolar degeneration of hepatocytes, necrosis of pancreatic cells and fatty changes in peripancreatic hepatocytes are the main lesions (Usha-Rani and Ramamurthi, 1989). The effects of cadmium on the kidneys and gills of the stickleback, *Gasterosteus aculeatus* has been studied by Oronsaye (1989). Cytological breakdown of kidneys and gill tissue has been observed. The renal tubules of silver carp *Hypophthalmichthys molitrix,* during cadmium toxicity, undergo swelling, atrophy and focal necrosis (Guo *et al.*, 1989). Morsy and Protasowicki (1990) have observed pathological alterations in the gill filaments and respiratory lamellae, hepatopancreas and kidney of common carp but the skin of the fish did not elicit any significant changes. A hepatic perivascular fibrosis in the eel, *Anguilla anguilla* has been reported by Lemaire and Lemaire (1992) after exposing them to a concentration of 5

µg Cd /l of sea water. In the juvenile stages of the eel, gills and liver are the major organs affected (Lemaire, 1993). While the lesions in gills are reversible, those in the liver are irreversible and affects not only the hepatocytes but also the blood and biliary tracts. Sövényi and Szakolczai (1993) have studied the histopathologic changes in the gills, kidney and liver of common carp, *Cyprinus carpio* exposed to different concentrations of Cd from 5 to 35 ppm for 96 hrs. Oedema of the secondary lamellae of the gills is the main finding. There is also a decrease in mucus cells. In kidneys, there is distension of Bowman's capsule and degeneration of renal tubules. Liver cells also undergo degeneration. Lipid peroxidation in the liver and kidney of *Channa punctatus* and *Clarias batrachus* on exposure to cadmium has been observed by Rana and Singh (1996).

The ability of cadmium to cause deformities in the olfactory system and lateral line sense organs is well established in many fishes. In *Anabas testudineus*, a sublethal concentration of cadmium (26 mg $CdCl_2$ /l) for 70 days has induced marked changes in olfactory epithelium (Chakrabarti *et al.*, 1993). The fusion and disruption of ciliated structure in the free border of epithelium rupture of the receptor and supporting cells, damage of microvilli on the nonsensory epithelium and fragmentation of microridges of the stratified epithelial cells were the microanatomical variations. Chakrabarti *et al.* (1994) have studied the microanatomical and histological alterations on the olfactory epithelium of the fish, *Mystus vittatus* after exposure to sublethal concentration of cadmium (20mg $CdCl_2$ /l) for 70 days. Fusion and disruption of linguiform process, necrosis of mucosal border of the olfactory lamellae, disruption of sensory receptor cells, obliteration of cilia on the apical surface of olfactory epithelium and damage of microvilli on the apex of the non-sensory epithelial cells are observed. The most distinct histological alterations are the rupture and the dissolution of cell membranes of supporting and receptor cells culminating in clumped nuclei, severe damage and shrinkage of receptor cells forming empty spaces, degeneration of basal cells and basement membrane, dilation of blood vessels and blood cells. Cadmium exposure reduces the dispersion of pigment in the integumentary melanophores of the crab *Uca pugilator* (Reddy and Fingerman, 1995). This decreased black pigment dispersion is apparently due to effects of cadmium on the neuroendocrine processes that control the melanophores. The neuroendocrine complex in the eyestalk is the source of a black pigment-dispersing

hormone (BPDH). Histological studies reveal that cadmium exposure results in depletion of the neurosecretory material in the eyestalks and brain. The olfactory system of wild brown trout (*Salmo trutta*) is found to be completely degenerated when they are exposed to water with cadmium concentrations more than that present in their habitat (Moran *et al.*, 1986). Severe damage to the lateral line and olfactory systems on exposure to cadmium was noticed in the migratory fish, *Galaxias fasciatus* juveniles by Baker and Montgomery (2001). However the changes are reversible.

The gonads and heart have also been reported to be affected by cadmium toxicity. Das (1988) has studied the effects of cadmium on the gonads of *Labeo bata* and observed that the testes of the treated fish are adversely affected and the germinal epithelium is mostly ruptured. In the ovary, the follicular development is arrested in oocyte-I and II stages and ovarian atresia and crumpling of larger oocytes are evidenced. The induction of myocardial edema by cadmium exposure has been suggested in the fish, *Halobatrachus didactylus* after histological interventions by Coucelo *et al.* (1998).

The actual targets of toxicity are molecules and these hits are closely followed by alterations in pathways, organelles and cell structure / function (Moore and Simpson, 1992; Braunbeck *et al.*, 1992). Specific histopathological parameters have been proposed as good indicators of heavy-metal pollution. Hence the study of ultrastructural alterations in target organs from sentinel animals after heavy-metal exposure may provide a useful tool in understanding the cellular role in heavy-metal metabolism (Rubio *et al.*, 1993).

Cadmium induced ultrastructural changes in the kidney of spot, *Leiostomus xanthurus* have been studied by Hawkins *et al.* (1980).The proximal tubule cells elicited an increase in heterogeneous bodies along with epithelial desquamation. Some mitochondria were contracted and dense while others were swollen with granular matrices and focal electron densities. Bowman's space of the renal corpuscle was swollen and often contained cellular debris. Deformities in the kidney tubules have been noticed in a fresh water fish, *Puntius sophore*, after exposing them to 0.1ppm $CdCl_2$ by Shrivastava and Pandey (1986). The cells of the kidney tubules increased in size, hypertrophied and obliterated the lumen.The

cytoplasm and nuclei were degenerated and vacuolated. The size of glomeruli also increases. The blood cells and plasma display degenerative changes and their staining capacity reduce considerably. In silvercarp kidney, cadmium causes severe ultrastructural changes (Guo *et* al., 1989). In *Thais haemastoma* cadmium toxicity was characterized by accumulation of membrane bound vesicles in digestive cells. These cells contain electron dense material and membrane debris. Basophilic cells show swelling of the rough endoplasmic reticulum vesicles. The calcium-containing cells seem to be unaffected by cadmium (Rubio *et al.*, 1993). The skin of cadmium exposed common carp, *Cyprinus carpio* has been studied at ultrastructural level by Iger *et al.* (1994). The basal lamina and the skin surface become highly undulating. Chloride cells appear between the pavement cells. Filament cells contain many electron-transparent and electron-dense secretory vesicles. Mitotic cells are commonly seen, mainly in cells adjacent to club cells or close to the epidermal surface. Mucous cells differentiate close to the skin surface, become elongated and synthesize highly electron-dense mucosomes. The epidermis becomes infiltrated by many leucocytes. As many of these changes are also associated with stressors other than cadmium, the specific changes for cadmium are the appearance of tumor like bodies at the skin surface, the appearance of Merkel cells throughout the epidermis and the coupling of leucocytes. Ultrastructural alterations in the gills of freshwater fish *Gnathonemus petersii* exposed to cadmium was studied by Alazemi *et al.* (1996). Formation of large subepithelial spaces within the secondary lamellae at a concentration of 1 mg/l and lamellar aneurism at 10mg/l are the major changes.

2.2.2. Effects on the Physiology of aquatic organisms

Generally, aquatic toxicological research is being applied at higher levels of biological organization like populations, communities and ecosystems to monitor environmental effects, conduct hazard assessments and make decisions of a regulatory nature (Mayer *et al.*, 1992; Varanasi *et al.*, 1992). But the fact is that ecologically important effects have already occurred at lower levels of biological organization by the time they are detected at the higher levels. So with a thorough understanding of the effects of the toxicants on various physiological indices, the problems can be detected before it affects the ecosystem as a whole. The

physiological indices generally affected by the pollutants include the haematological parameters, enzymes, serum proteins, glucose and glycogen levels, cholesterol and hormones.

2.2.2.1. Haematology

Cadmium causes marked changes in various haematological parameters in aquatic organisms. Significant reductions of hematocrit, hemoglobin and red blood cell count with significant increase in lymphocytes were found in the cadmium-exposed flounders *Pleuronectes flesus* (Sjobeck and Larsson, 1978). In a field investigation on perch, *Perca fluviatilis* from a cadmium contaminated river, Sjöbeck *et al.* (1984) have reported that the lymphocyte count is 45-100% higher than those from reference habitats. A slight anaemia is also noticed in these fishes. A dose of 24 ppm of cadmium nitrate for 90 hrs causes significant decrease in erythrocyte count, hematocrit and hemoglobin content and an increase in erythrocyte sedimentation rate (ESR) in *Cyprinus carpio* (Beena & Viswaranjan, 1987). However, the leucocyte count, thrombocyte count and blood clotting time did not significantly change due to the exposure. Effect of 24-hour LC_{50} concentrations of $CdCl_2$ on erythrocyte and its related parameters in *Anabas testudineus* has been studied by Banerjee & Kumari (1988). No significant change is noticed in shape, length, breadth and erythrocyte surface area. Nucleus becomes oval from almost round shape. Total erythrocyte count, haemoglobin content and packed cell volume (PCV) decrease significantly whereas ESR, mean corpuscle volume (MCV) and mean corpuscle haemoglobin (MCH) increase significantly. Morsy and Protasowicki (1990) have observed that, cadmium bioaccumulation significantly raises erythrocytes count, hemoglobin content, hematocrit value and blood glucose, but decreases leukocyte count in *Cyprinus carpio* L. when exposed to acute concentration of cadmium (0.5 mg Cd /dm^3 water) for 24 hrs. Tort *et al.* (1990) have exposed dogfish, *Scyliorhinus canicula* to a cadmium concentration of 50 ppm for 1, 2, 3 and 4 days and no differences were found in majority of haematological parameters, except for the significant increase in red blood cell counts. In *Anabas testudineus* a sublethal concentration of cadmium for 30, 45 and 60 days evoked a significant increase in erythrocytes count, leucocyte count, and haemoglobin(Hb) concentration (Saravanan and Natarajan, 1991). Allen (1993) has studied the effects of acute exposure to cadmium chloride on the

haematological profile of *Oreochromis aureus* (Steindachner). Plasma osmolality is the most sensitive blood parameter affected before other parameters change and cadmium does not depress erythrocyte counts. Cadmium chloride, both at weak and strong dose levels, produces haematological abnormalities in *Tilapia mossambica* (Aziz *et al.*, 1993). An increase in leucocyte count, erythrocyte count, Hb, PCV and MCV have been recorded at a strong dose of cadmium chloride (10 µg /15 1) for 2 days. On extending this exposure for 7 days Hb, leucocyte count, erythrocyte count and MCH increase, whereas PCV and MCV decrease. Fish exposed to weak dose for a period of 7 days (2.5µg /15 l) have shown an increase in Hb, leucocyte count, erythrocyte count, PCV, MCV & MCH. Mukherjee and Sinha (1993) have observed a marked decrease in Hb, haemtocrit(Hct) value and total erythrocyte count, along with an increase in MCV and MCH after 2 weeks of cadmium exposure in *Labeo rohita*. In the American eel, *Anguilla rostrata*, after 8 weeks of exposure to 150 µg Cd /l, there was a significant reduction in the total erythrocyte count, Hb, and Hct. Total leukocyte counts, leukocrit, and large lymphocytes were significantly increased, while the proportion of small lymphocytes falls (Gill and Epple, 1993). Changes in erythron organization on exposure to cadmium have been studied in gold fish, *Carassius auratus* by Houston *et al.* (1993). In fish exposed to 11% of LC_{50}, total cell numbers and the incidence of cell division decreased while karyorrhexis increased. At a sublethal concentration of cadmium the total leucocyte count increases in the fish, *Channa punctatus* (Sastry and Sachdeva1994) where as total erythrocyte count decreases (Bala *et al.*, 1994). The major carp, *Catla catla* shows a drastic decline in total erythrocyte count, Hb and PCV on exposure to cadmium (Vincent *et al.*, 1996). Thus, the haematological alterations due to cadmium toxicity cannot be generalized and is highly variable between as well as within species.

2.2.2.2. Enzymes

There are indications of depressed or accelerated enzyme activity in aquatic organisms exposed to low concentrations of metals (Hewitt and Nicholas, 1963). Cadmium is a toxic, non-essential heavy metal inhibiting numerous enzymes with functional sulfhydryl groups (Reddy *et al.*, 1989). The effects of cadmium on the enzymes of various Indian and exotic species of fishes have been studied.

Jackim (1974) has studied the effect of cadmium on six enzymes, xanthine oxidase, acid phosphatase, alkaline phosphatase, catalase, $Na^{+}K$ ATP- ase and Mg ATP-ase in *Fundulus heteroclitus* and observed a reduction in activity of all enzymes except $Na^{+}K$ ATP ase. The enzyme, alpha glycero-phosphate dehydrogenase found in trout muscle is inhibited by cadmium (Bergmann and Brown, 1974). Hilmy *et al.* (1985) have studied the in vivo and in vitro effects of cadmium exposure on five enzymes of *Mugil cephalus* and found that gill aspartate amino transferase and alanine amino transferase are the most sensitive enzymes whereas liver alkaline phosphatase and heart lactic dehydrogenase show maximum inhibition at higher cadmium concentrations. In the catfish, *Heteropneustes fossilis*, after exposure to 0.26 mg /l of cadmium for 60 days, acid phosphatase activity is inhibited in liver and ovary but increased in kidney and intestine while activity of alkaline phosphatase decreased in liver, kidney and intestine but increased in ovary and muscles (Sastry and Subhadra, 1985). In plaice, *Pleuronectes platessa* cadmium inhibits ethoxyresorufin O-deethylase, glutathione S-transferase and glutathione peroxidase (George and Young, 1986). Cadmium exposure produces significant alterations in the levels of glucose, aspartate aminotransaminase, and alanine aminotransaminase in freshwater fish *Notemigonus crysoleucas* (Benson *et al.*, 1987). Cadmium induced inhibition of the LDH activity in the blood, bile, liver and kidney of O. *mossambicus* has been reported by Gao,-Zhenpan (1987). The interaction of Cd^{2+} with the plasma membrane Ca^{2+} -transporting ATPase of fish gills has been studied by Verbost *et al.* (1988). They are of the opinion that the inhibition by Cd^{2+} occurs directly on the Ca^{2+} binding site of the Ca^{2+} transporting ATPase and the Cd^{2+}- induced inhibition of Ca2+-transporting enzymes is the primary effect in the Cd^{2+} toxicity towards cells followed by several secondary effects due to a disturbed cellular Ca^{2+} metabolism. The hepatic alkaline phosphatase, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and lactate dehydrogenase (LDH) activities increase, while amylase activity decrease after cadmium exposure in the fish *Cirrhinus mrigala* (Shakoori *et al.*,1990). In scorpionfish (*Scorpaena guttata*), the exposure to sublethal levels of cadmium results in the inhibition of Cu-Zn-superoxide dismutase (SOD) activity in the intestine (Bay *et al.*, 1990). The sublethal concentration of cadmium produce a decrease in alkaline phosphatase activity in the brush border and columnar epithelial cells of intestine and intestinal caeca of *Notopterus notopterus* (Ghosh and Chakrabarti,1991). The

enzymes in rosy barb, *Barbus conchonius* shows a varied response to cadmium toxicity between in vivo and in vitro exposures (Gill *et al.*, 1991). Acid phosphatase, GOT and GPT are inhibited where as alkaline phosphatase is stimulated in vivo and in vitro. LDH activity increases in vivo but decreases in vitro. The activities of biotransformation enzymes, ethoxyresorufin-0-dealkylase, pentoxyresorufin-0-dealkylase and glutathione S-transferase are found to be lowered in two species of fishes (painted comber, *Serranus cabrilla* and striped mullet, *Mullus barbatus*) collected from a cadmium polluted area in North West Mediterranean (Romeo *et al.*, 1994). Sublethal concentration of cadmium increases GPT and GOT levels in the serum of *Channa punctatus* (Sastry and Sachdeva, 1994). Cadmium induced changes in ion level and ATPase activity in the muscle of the fry and fingerlings of common carp, *Cyprinus carpio* has been studied by Suresh *et al.* (1995). At lethal concentrations, all the ion levels and ATPase activity progressively decreased over time of exposure. On long-term exposure at sub lethal concentrations, an initial decrease followed by an increase in all the parameters is noticed. In another study in common carp, exposure to Cd at 20ppm, lowers the activity of SOD in the erythrocytes where as that of CAT and plasma GOT and GPT increased (Zikic *et al.*, 1997). The activities of acetylcholinesterase, alkaline phosphatase and glutathione S-transferase were found to be inhibited in *Tilapia nilotica* living in cadmium contaminated areas (El Demerdash and Elagamy, 1999). In the same fish, sublethal concentrations of Cd caused a decrease in the activities of phosphofructo kinase, lactate dehydrogenase and creatine kinase in white muscle where as in red muscle, phosphofructokinase and LDH increases (Almeida *et al.*, 2001). In *Carassius auratus,* cadmium exposure elevates the GOT, GPT and superoxide dismutase in erythrocytes (Zikic *et al.*, 2001).

In the shrimp, *Callianassa tyrrhena*, cadmium inhibits glutathione S-transferase and esterase activity in vitro where as in vivo, cadmium caused a dose dependent increase in activity of the above enzymes (Thaker and Haritos, 1993). In the mediterranean white mussel, *Donax trunculus*, activities of six enzymes (cytochrome oxidase, lactate dehydrogenase, malate dehydrogenase, ATPase, alkaline phosphatase and alpha –amylase) on exposure to 0.1, 1 and 10 ppm of Cd has been studied by Mizrahi et.al. (1993). Cytochrome oxidase is the most sensitive enzyme while alpha -amylase, ATPase and alkaline phosphatase were only slightly

affected. A decrease in the activity of lactate dehydrogenase, succinate dehydrogenase and malate dehydrogenase and an increase in acid and alkaline phosphatase activities were observed in the crab, *Scylla serrata*, exposed to 2.5ppm of $CdCl_2$ (Reddy and Bhagyalakshmi, 1994). Also an increase in protease, alanine aminotransaminase, asparate aminotransaminase, glutamate dehydrogenase, AMP deaminase, adenosine deaminase, arginase and glutamine synthetase is observed. A significant decrease in succinate dehydrogenase activity and a significant elevation of LDH activity are noticed in the gill and muscle of marine bivalve *Perna viridis* during cadmium exposure (Arasu and Reddy, 1994).

2.2.2.3. Other Physiological indices

Some of the other biochemical indices of physiological importance include the serum and muscle protein levels, tissue glycogen, blood glucose, cholesterol, different hormones, total carbohydrate, lactate and pyruvate. Cadmium induces marked alterations in many of these parameters in various species of fishes and shellfishes.

Total protein content in cadmium exposed *Mugil cephalus* shows an increase in the liver, gill and serum while there was no change in heart protein (Hilmy *et al.*, 1985). Cadmium toxicity in *Clarias batrachus* causes an increase in the protein content of liver, kidney, stomach, intestine, testis and ovary, where as in the muscle a decrease was observed (Jana and Sahana, 1988). Shakoori *et al.* (1990) have observed a decrease in the concentration of cholesterol, glycogen and free amino acids (FAA) in *Cirrhinus mrigala* as a result of cadmium toxicity. In case of plasma cortisol and glucose values, an initial increase which returns to normal values on long term exposure suggesting the capacity of tilapia to adapt to low levels of cadmium (Pratap and Bonga, 1990). Glycogen, total protein and cholesterol in the ovary of hill stream teleost, *Garra mullya* (Sykes) decreases on exposure to cadmium while liver glycogen remain unaltered (Khan *et al.*, 1991). In common carp, *Cyprinus carpio*, cadmium toxicity caused a significant decrease in the blood plasma electrolytes (Kuroshima, 1992). In *Labeo rohita,* cadmium produces a decrease in plasma protein, liver protein and glycogen content of liver and muscles. It also causes an

increase in blood glucose (Mukherjee and Sinha, 1993). The serum proteins in *Tilapia mossambica* displayed different responses at strong and weak doses of cadmium. An initial decrease followed by an increase at strong dose whereas a steady increase at weak dose was noticed (Aziz *et al.*, 1993). A decrease in plasma proteins was observed in *Channa punctatus* exposed to a sublethal concentration of cadmium (Sastry and Sachdeva, 1994). Cadmium depresses the oogenesis in brown trout but will not affect the fertilization and development of eggs produced (Brown *et al.*, 1994). In rainbow trout, *Oncorhynchus mykiss*, cadmium delays growth hormone expression (Jones *et al.*, 2001) and also inhibits unidirectional Ca uptake from water into the gill thereby inducing hypocalcemia (Zohouri *et al.*, 2001). In nile tilapia, *Oreochromis niloticus*, different doses of cadmium decreases glycogen content of white muscle. It also reduces the glucose uptake in white muscles where as in red muscles glucose uptake increases (Almeida *et al.*, 2002).

Filtration rates of bivalves are drastically reduced on exposure to cadmium (Mohan *et al.*, 1986). The effect of cadmium on oxygen uptake in the brown mussel, *Perna indica* has been studied by Baby and Menon (1986) and they are of the opinion that at sublethal levels, cadmium functions as a respiratory depressant. The rate of filtration in *Villorita cyprinoides* decreases exponentially with increasing concentrations of cadmium (Abraham *et al.*, 1986). Reddy and Bhagyalakshmi (1994) have studied the changes in oxidative metabolism in hepatopancreas, muscle, and hemolymph of the edible crab *Scylla serrata*, at a sublethal concentration (2.5 ppm) of cadmium chloride. Glycogen, total carbohydrate and pyruvate decrease whereas lactate levels in hepatopancreas and muscle increases. Lysosomal enzymes in the digestive glands of the blood clam *Anadara granosa* vary significantly on exposure to cadmium ion (Anthony and Patel, 1996). The fresh water mussel, *Lamellidans corrianus* on exposure to cadmium shows a reduction in the tissue glycogen levels in the gills and hepatopancreas (Rajalekshmy ands Mohandas, 1993).

3. MATERIAL AND METHODS

Experiments were conducted at Krishi Vigyan Kendra (KVK) of Central Marine Fisheries Research Institute (CMFRI), Narakkal, Ernakulam district. Studies on histopathology and ultrastructure, blood and serum samples were carried out at CMFRI Head Quarters, Cochin.

3.1. EXPERIMENTAL FISH

Pearl spot, *Etroplus suratensis* (average weight 35 ± 5g) collected from the brackish water ponds of KVK, were used in the present study. Experiments were conducted in 1 ton capacity oval FRP tanks holding pressure filtered (TRITON – II sand filter, USA) brackish water (salinity 20 ± 2 ppt) with continuous aeration.

3.2. EXPERIMENTAL DESIGNS

3.2.1. Aflatoxicosis

Two groups of fishes were maintained in triplicate with ten fishes per tank. One group (control) was fed on formulated pelleted feed having 38 % crude protein and the other group (treatment) on formulated feed of same composition but incorporated with aflatoxin B_1 (AFB_1) for a period of 8 weeks. Sampling was done at fortnightly intervals by removing six fishes from both groups and samples of blood and tissues of vital organs (viz, liver, kidney, spleen and thymus) were collected for haematological, histological and ultrastructural investigations. At the end of the experiment, immunomodulatory effects of aflatoxin were assayed by testing the skin sensitivity to Phytohaemagglutinin (PHA) and antibody titre against *Aeromonas hydrophila*.

3.2.2. Cadmium Toxicity

Cadmium was chosen for testing heavy metal toxicity. The 96 hour LC_{50} of cadmium was determined and one-tenth (1/10) of this value was taken as the sublethal dose for chronic toxicity studies.

Two groups of fishes were maintained in triplicate with ten fishes per tank. One group served as the control and the other group (treatment) was exposed to cadmium at sublethal concentration for a period of 8 weeks. Six fishes per group were sampled at fortnightly intervals. Samples of blood and tissues of liver, kidney, spleen, thymus and gills were collected for haematological, histological and ultrastructural investigations.

3.3. PRODUCTION OF AFLATOXIN

Aflatoxin was produced by the method of Shotwell *et al.* (1966). Pure strain of *Aspergillus flavus* obtained from Central Food Technology Research Institute, Mysore, was mass cultured on rice. The toxin thus produced was extracted according to Pons and Goldblatt (1969) and got quantified from the laboratory of Spices Board, Ministry of Commerce, Government of India, Palarivattom , Ernakulam district, Kerala.

3.4. FEED PREPARATION

3.4.1. Normal feed

A balanced feed with 38% crude protein was formulated as shown below.

Ingredient	% Inclusion
Fish meal	21.5
Soy flour	32.0
Wheat flour	30.0
Cod liver oil	6.00
Gelatin	5.00
Guargum	2.00
Mineral mix	2.00
BHT	1.50

Vitamins were added in excess after gelatinisation by steam cooking followed by pelletisation and drying.

3.4.2. Aflatoxin-incorporated feed

The feed formula and ingredients were same as that of normal feed. AFB_1 dissolved in chloroform was added to the dough after gelatinisation (0.4mg /Kg feed), mixed thoroughly and pelletised.

3.5 DETERMINATION OF LC_{50} FOR CADMIUM

LC_{50} for Cadmium was determined as per the method of Reish and Oshida (1987) with slight modifications. After the range finding assay, triplicate sets of six fishes at each dose were maintained for 96 hrs and the mortality was recorded. The data analysis for determining LC_{50} was done as per Mohapatra and Saha (2000).

3.6. CHRONIC TOXICITY STUDY WITH CADMIUM.

In the treatment group, measured quantity of cadmium chloride stock solution was added so that the final cadmium concentration was 9.4 ppm (one tenth of LC_{50}) in the water. The fishes were fed on a formulated pelleted feed with 38% crude protein as described under section 3.4.1. Daily 50 % of the water was exchanged along with the removal of faecal matter and excess feed. On sampling days six fishes from both groups were removed for collecting blood along with tissues for haematology, histopathology and electron microscopy.

3.7. BLOOD COLLECTION FROM FISH

Blood samples collected from 6 fish per group were pooled and used for haematological investigations. Fishes were anaesthetized using Benzocaine prior to bleeding. The anticoagulant used was Heparin sodium (BEPARINE , Biological E. Limited, Hyderabad, India). The blood samples were collected by heart puncture using heparinised syringe with 26-gauge needle and transferred to heparinised vials. The blood thus collected was transported immediately to the laboratory in icebox for further analysis.

3.8. HAEMATOLOGY

All the haematological parameters were estimated as per Schaperclaus (1986).

3.8.1. Erythrocyte count and Leukocyte count.

Diluted blood was loaded into the Neubauer counting chamber and erythrocytes were counted in the five group squares (1group square=16 small squares). The total erythrocyte count (EC) was determined as follows:

$$EC/mm^3 = \frac{\text{Erythrocytes in eighty small squares}}{\text{Area} \times \text{depth of chamber} \times \text{dilution}}$$

The leucocytes were counted in the four large squares at the four angular points of the Neubauer counting chamber. The total Leucocyte count (LC) was calculated as follows:

$$LC/mm^3 = \frac{\text{No of leucocytes in four squares of } 1mm^2}{\text{Area} \times \text{depth of chamber} \times \text{dilution}}$$

3.8.2. The Erythrocyte Sedimentation Rate and Packed Cell Volume

Erythrocyte Sedimentation Rate (ESR) and Packed Cell Volume (PCV) were determined using commercially available ESR tubes (Wintrobes tubes)

Blood with the anticoagulant was filled in the heparinised ESR tube up to the 0 - mark and kept vertically. The sedimentation rate was read out after one and two hours.

The ESR tube with the blood was subjected to centrifugation at 3000 rpm for 3 min. The packed cell volume was directly read out from the graduated ESR tube and expressed as percentage.

3.9. SERUM FACTORS

The blood intended for serum separation was allowed to clot by keeping the vials in a slanting position at room temperature for 1 hr after which it was kept at 4^0 C for the clot to contract. The clot was then removed by centrifugation at 3000 rpm for 15 min. The serum factors analysed include Alkaline phosphatase, Aspartate amino Transaminase (AST)/ SGOT, Alanine amino Transaminase(ALT)/ SGPT, Total proteins, Albumin, Globulin and Albumin:Globulin ratio. OD was measured using Genesys-10 UV/ VIS Spectrophotometer (Thermospectronics, USA).

3.9.1 Alkaline Phosphatase

The serum alkaline phosphatase activity was assayed using a commercially available diagnostic kit (Sigma Diagnostics, India, Pvt. Ltd. Product no. 72011) according to Kind and King (1954). The O.D of the samples were recorded at 510 nm.

3.9.2. Aspartate amino Transaminase (AST) / SGOT

AST activity was assayed using a commercially available diagnostic kit (Sigma Diagnostics, India, Pvt. Ltd. Product 72141) according to Reitman and Frankel (1957). The absorbance was measured at 505 nm.

3.9.3. Alanine amino Transaminase(ALT) / SGPT

ALT activity was assayed using a commercially available diagnostic kit (Sigma Diagnostics, India, Pvt. Ltd. Product no.72151) according to Reitman and Frankel (1957). The absorbance was measured at 505 nm.

3.9.4. Total Protein and Albumin

Total protein and albumin were determined by the Biuret method and BCG dye binding method respectively using a commercially available diagnostic kit (Sigma Diagnostics, India, Pvt. Ltd. Product no. 72111, 72121). The absorbance was

measured at 555nm for total proteins and 630 nm for albumin. Globulins were determined by subtracting the albumin value from total protein value.

3.10. HISTOPATHOLOGY

Tissues of liver, kidney, spleen, thymus and gills were taken for histopathological investigations.

3.10.1. Fixation

The tissues were fixed in 10 % buffered formalin overnight. The tissues were scored with a sharp blade for easy penetration of the fixative.

3.10.2. Tissue Processing And Microtomy

Tissues were processed before paraffin embedding in an automatic tissue processor (Leica, Germany) and sections of 5µ thickness were cut in a semi automatic rotary microtome (Leica, Germany). The alcohol gradient is given in Annexure-I

3.10.3. Staining

Paraffin sections were cleared in xylene, hydrated with descending grades of alcohol and stained in haematoxylin after which they were passed through acid alcohol, Scott's tap water and eosin. The stained sections were dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX.

3.11. TRANSMISSION ELECTRON MICROSCOPY

Ultrastructural studies were carried out in Hitachi – H-600 Transmission Electron Microscope (HITACHI Ltd, Tokyo, Japan). The tissue preparation and processing was done as per Dawes (1988). Vital organs like liver, kidney, spleen and thymus were analysed for ultrastructural alterations.

3.11.1. Fixation

One mm cubes of tissues from vital organs were excised from anaesthetized fishes and immediately transferred to chilled buffered 3% gluteraldehyde solution in cacodylate buffer, pH 7.3. The tissues were fixed for 3 hrs at 4^0 C. The fixed tissues were washed in 0.1M sodium cacodylate buffer three times (30 min each) and kept overnight. Tissues were post fixed in 1% osmium tetroxide (in cacodylate buffer) for 2 hrs at 4^0 C. The tissues were again washed 3 times (30 min each) in buffer.

3.11.2. Dehydration and Embedding

Dehydration of tissues were carried out in ascending grades of acetone (Annexure-II) at 4^0 C and embedded in Spurr's resin as per the method described by Spurr (1969).

3.11.3. Sectioning and Staining

Ultra thin sections (60-90 nm) were taken in an LKB Nova Ultra microtome (LKB – Producter AB, Sweden) using glass knives. These sections were lifted on to matted surface of copper grids (300-mesh size). The sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshally, 1965), dried and observed under transmission electron microscope.

3.12. IMMUNOLOGICAL STUDIES

These tests were performed only in fishes from the aflatoxicosis experiment.

3.12.1. Skin sensitivity to Phytohaemagglutinin (PHA)

Skin sensitivity to the T- cell mitogen, PHA was determined according to Rajan *et al* (1986). Six fishes from the control and treatment groups were taken for performing this test. 0.1 ml of PHA-M was injected intradermally at the caudal

peduncle of the fishes and the skin thickness at the caudal peduncle was measured using a screw gauge after 24, 48 and 72 hrs.

3.12.2. Antibody Titre

Antibody titre against *Aeromonas hydrohila* was assayed. Six fishes each from the treatment and control group were injected with formalin-killed *Aeromonas hydrohila* at 10^7 cells/ml. A second injection of the same dose was given on the 6^{th} day and serum collected after 2 weeks. Agglutination test was performed using Tarsons microtitre agglutination plates (96 wells) according to Karunasagar and Karunasagar (1993).

The antigen suspension was prepared by formalin treatment of *Aeromonas hydrohila* cells and adjusting its density to about 10^9 cells /ml. In each well of the micro titre plates 50 µl of saline was taken. Serial doubling dilutions of the serum were prepared by adding 50 µl of serum to the 1^{st} well, mixing and transferring 50 µl to the 2^{nd} well and so on, making the dilution of antibody in the 1^{st} well as 1:2; in the 2^{nd} well as 1:4 and so on. A saline control was also maintained to check for auto agglutination of the antigens. To each well 50 µl of the bacterial suspension was added and the plates were incubated overnight at room temperature in a moist chamber. Clumping or agglutination of bacterial aggregates was taken as positive reaction and button formation as negative.

3.13. STATISTICAL ANALYSIS

Data from the studies on haematological parameters and serum factors were subjected to statistical analysis. Mean value of each parameter from the treatment group was compared with that of the control group on every sampling day using students' t -test.

4. RESULTS

4.1. STUDIES ON AFLATOXICOSIS

4.1.1. Gross Pathology

There was no marked variation in the behaviour of aflatoxin fed fishes from the control group. However the liver showed gross changes from second week of post feeding. Initially the livers appeared pale. On termination of the experiment after eight weeks, the livers from the treatment group contained numerous black spots and raised pale patches. The other internal organs did not elicit much variation. (Plates 1 – 6)

4.1.2. Haematology

Total erythrocyte count, total leucocyte count, erythrocyte sedimentation rate (ESR), and packed cell volume (PCV) were determined from the treatment group and control group on all sampling days viz, 14^{th}, 28^{th}, 42^{nd} and 56^{th} day of the rearing period. The mean values of total erythrocyte count, leucocyte count, ESR and PCV from treatment and control groups on the four sampling days are presented in table 1.

4.1.2.1. Total Erythrocyte Count

The total erythrocyte count in the treatment group was significantly ($P<0.01$) higher than the control from 28^{th} day onwards whereas initially, on 14^{th} day, it registered a lower value than the control.

4.1.2.2. Total Leucocyte Count

Total leucocyte count was not affected till the 14^{th} day of the experiment. However, it was significantly ($p<0.01$) higher in treatment than control on 28^{th}, 42^{nd} and 56^{th} day of the experiment.

4.1.2.3. Erythrocyte Sedimentation Rate (ESR)

The ESR varied significantly ($p<0.01$) between control and treatment from 14^{th} to 42^{nd} day of the experiment. It was higher in the treatment group than control on 14^{th} and 42^{nd} day whereas on 28^{th} day, the ESR was lower in the treatment group when compared to control.

Plate 1. *E suratensis* with exposed abdomen exhibiting the viscera of control group

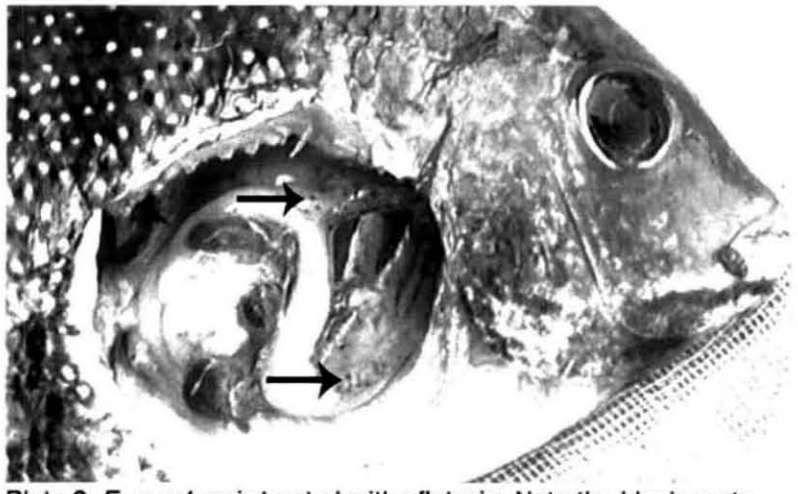


Plate 2. *E. suratensis* treated with aflatoxin. Note the black spots on the liver surface

Plate 3. *E. suratensis* treated with aflatoxin exhibiting pale liver.

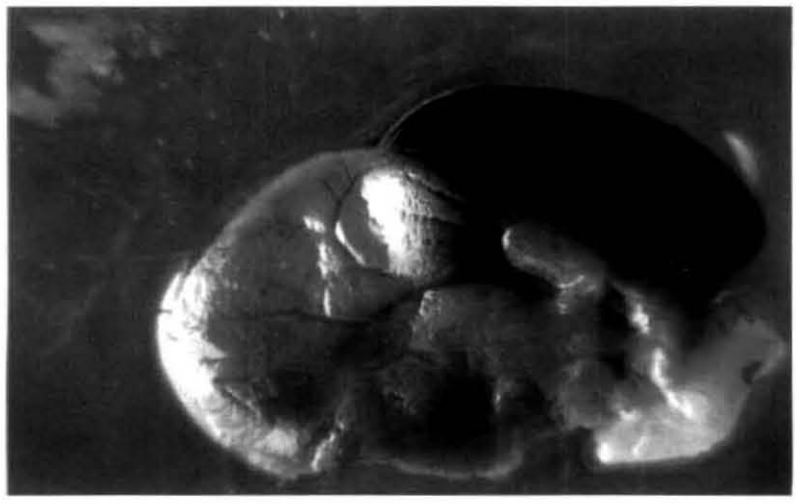


Plate 4. Liver of the control group *E.suratensis*.

Plate 5. Liver with pale patches from fishes treated with aflatoxin for 8 weeks

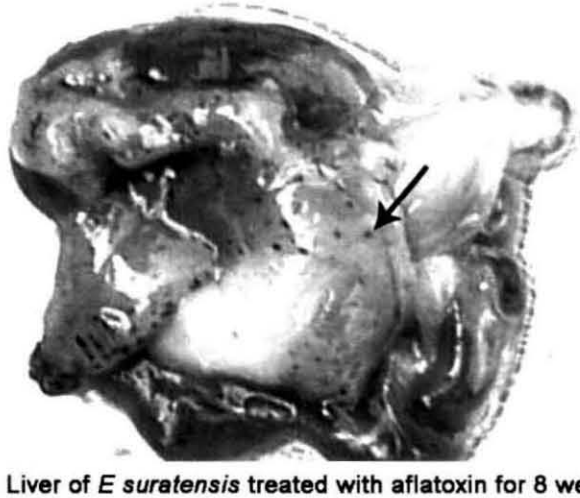


Plate 6. Liver of *E suratensis* treated with aflatoxin for 8 weeks. Note the black spots on the surface.

Table 1. Effect of aflatoxin on the haematological parameters of *E. suratensis*

Total erythrocyte count ($\times 10^6/mm^3$)		
Days	treatment	control
14 *	8.61±0.14	9.13±0.218
28 **	6.84±0.126	4.46±0.075
42 **	8.34±0.111	4.38±0.095
56 **	6.51±0.1	5.12±0.096

Total leucocyte count ($\times 10^3/mm^3$)		
Days	Treatment	Control
14	57.67±1.26	57.33±1.53
28 **	61.83±1.76	28.00±2.29
42 **	66.00±2.00	30.00±1.32
56 **	42.33±2.02	28.67±2.52

ESR (mm/2hr)		
Days	Treatment	Control
14 **	19.67±0.577	15.33±1.528
28 **	5.67±1.155	11.33±2.082
42 **	11.67±0.577	5±1
56	5.33±0.577	7.33±1.155

PCV (%)		
Days	Treatment	Control
14	21.67±1.155	22.67±0.577
28	26.33±1.528	26±1
42 **	31.67±0.577	26±1
56 **	32.33±0.577	20.67±1.155

The mean values from treatment and control groups on each sampling day were compared by students' t-test.

* $p < .05$

** $p < .01$

4.1.2.4. Packed Cell Volume (PCV)

The PCV elicited significant ($p<0.01$) variations only at the later stages of the experiment (viz, on days 42 and 56). It was higher in the aflatoxin exposed fishes than the control group.

4.1.3. Serum Factors

The serum factors studied include total proteins, albumin, globulin, albumin:globulin ratio and the enzymes, alkaline phosphatase, aspartate aminotransaminase (AST) and alanine aminotransaminase(ALT). The mean values of serum proteins and enzymes from control and treatment groups on the four sampling days are presented in tables 2 and 3 respectively.

4.1.3.1. Total Protein

The total serum protein levels were significantly ($p<0.01$) lower in aflatoxin treated fishes on 28^{th}, 42^{nd} and 56^{th} day of the experiment. Although on the 14^{th} day it was higher in treatment than control, it was not statistically significant. The decrease in serum proteins due to aflatoxin is depicted in figure1.

4.1.3.2. Total Albumin

The serum albumin in the treatment group was lower than the control from 28^{th} to 56^{th} day of the experiment. However, initially, on the 14^{th} day it was higher in the treatment group than the control group

Table 2. Changes in serum proteins of *E. suratensis* treated with aflatoxin

Total protein (g%)		
Days	Treatment	Control
14	2.78±0.116	2.55±0.111
28 **	1.930±0.125	3.000±0.151
42 **	2.270±0.116	3.100±0.134
56 **	0.720±0.142	1.730±0.099

Albumin (g%)		
Days	Treatment	Control
14 **	1.198±0.056	0.805±0.056
28 **	0.366±0.067	1.476±0.074
42 *	0.338±0.060	0.528±0.074
56 **	0.089±0.041	0.306±0.060

Globulin (g%)		
Days	Treatment	Control
14	1.586±0.171	1.741±0.166
28	1.563±0.192	1.527±0.225
42 *	1.929±0.176	2.564±0.208
56 *	0.635±0.160	1.421±0.040

A/G ratio		
Days	Treatment	Control
14 *	0.764±0.117	0.467±0.075
28 **	0.240±0.075	0.986±0.196
42	0.178±0.048	0.209±0.047
56	0.154±0.107	0.215±0.036

The mean values from treatment and control groups on each sampling day were compared by students' t-test.

* $p < .05$

** $p < .01$

Table 3. Changes in serum enzyme profile of *E. suratensis* treated with aflatoxin.

AST /SGOT(enzyme units/ml)		
Days	Treatment	Control
14 **	8.052±2.577	19.736±1.728
28	2.21±1.306	2.495±2.612
42 *	6.2±1.959	0.073±1.076
56	0.5±0.494	00.00

ALT/SGPT (enzyme units/ml)		
Days	Treatment	Control
14	12.438±1.574	15.676±1.288
28	9.295±1.438	2.819±2.144
42 **	9.486±1.591	0.629±1.309
56	1.771±1.03	00.00

Alkaline phosphatase (KA units/ml)		
Days	Treatment	Control
14 **	3.66±0.323	1.3±0.32
28 **	4.39±0.346	2.47±0.472
42 **	14.72±0.42	3.99±0.375
56 **	21.09±0.452	17.76±0.498

The mean values from treatment and control groups on each sampling day were compared by students' t-test.

* $p < .05$

** $p < .01$

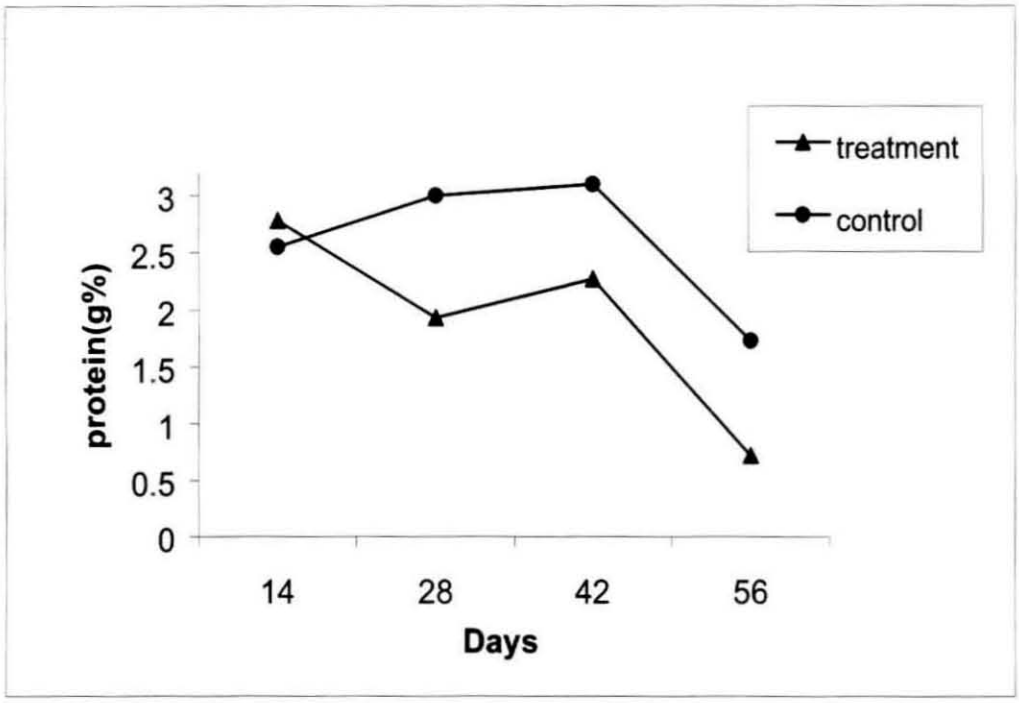


Figure 1. Effect of aflatoxin on the serum protein of *E. suratensis*

4.1.3.3. Total Globulin

The total globulin varied significantly ($p<0.05$) between control and treatment groups only on the 42nd and 56th day of the experiment. On these days the total globulin was lower in aflatoxin-treated fishes than the control group.

4.1.3.4. Albumin: Globulin Ratio (A/G Ratio)

The A/G ratio was affected only in the first two fortnights of aflatoxin treatment. It was significantly ($p<0.05$) higher in treatment than control on the 14th day where as on 28th day it was significantly ($p<0.01$) lower in treatment when compared to control. In the later stages of the experiment viz on days 42 and 56, the A/G ratio did not elicit remarkable variations.

4.1.3.5. Alkaline phosphatase

The serum alkaline phosphatase activity was significantly ($p<0.01$) higher in treatment than control throughout the experimental period. The mean enzyme activity (in KA units) in treatment group was 3.66,4.39,14.72 and 21.09 on 14th, 28th,42nd and 56th day respectively whereas that of control group was 1.3, 2.47, 3.99 and 17.76 on 14th, 28th,42nd and 56th day respectively.

4.1.3.6. Aspartate aminotransaminase (AST) / SGOT

The serum AST values elicited marked difference between the control and treatment groups only on 14th and 42nd day of the experiment. In the treatment group the enzyme activity was significantly ($p<0.01$) lower than control initially on the 14th day, where as it was significantly ($p<0.05$) higher than the control group on 42nd day of the experiment. The general trend was an increase in AST activity in aflatoxin-exposed fishes at the later stages of the experiment.

4.1.3.7. Alanine aminotransaminase (ALT) / SGPT

The serum ALT activity significantly ($p<0.01$) varied between the control and treatment groups only on the 42nd day of the experiment and it was higher in the

treatment group than the control. However, the general trend was an increase in the enzyme activity when compared to the control as the exposure time increased.

4.1.4. Immunological Studies

The immunologcal indices assayed include sensitivity to Phytohaemagglutinin (PHA) and antibody titre against *Aeromonas hydrophila*. These tests were carried out on termination of the rearing experiment.

4.1.4.1. Sensitivity to Phytohaemagglutinin (PHA)

The mean increase in skin thickness after 24, 48, 72 and 96 hrs after PHA injection in control and treatment groups is depicted in table 4. There was significant ($p<0.05$) difference between the control and treatment groups after 72 hrs. The mean increase in skin thickness was lower in the treatment group. The lowered PHA response in aflatoxin treated fishes is evident from figure 2.

4.1.4.2. Antibody Titre

The antibody titre against *Aeromonas hydrophila* was assayed. The mean titres after 2 weeks of injection were 74.67± 48.88 and 85.33 ± 36.95 for the treatment and control groups respectively. Although the titre was low in the treatment group, it was not statistically significant.

4.1.5. Histopathological Studies

The vital organs liver, kidney, spleen and thymus were studied for histological alterations after aflatoxin teatment.

4.1.5.1. Liver

In control animals the liver parenchyma was formed of the hepatic cells arranged in irregular glandular pattern with sinusoids and biliary canaliculi between

Table 4. Immune response of *E. suratensis* to the T- cell mitogen, Phytohaemagglutinin (PHA)

PHA - SENSITIVITY		
Mean increase in skin thickness (cm)		
Time after injection (hrs)	Treatment	Control
24 hrs	0.04 ± 0.027	0.042 ± 0.031
48 hrs	0.0475 ± 0.034	0.085 ± 0.039
72 hrs*	0.04 ± 0.027	0.11 ± 0.036

* $p < .05$

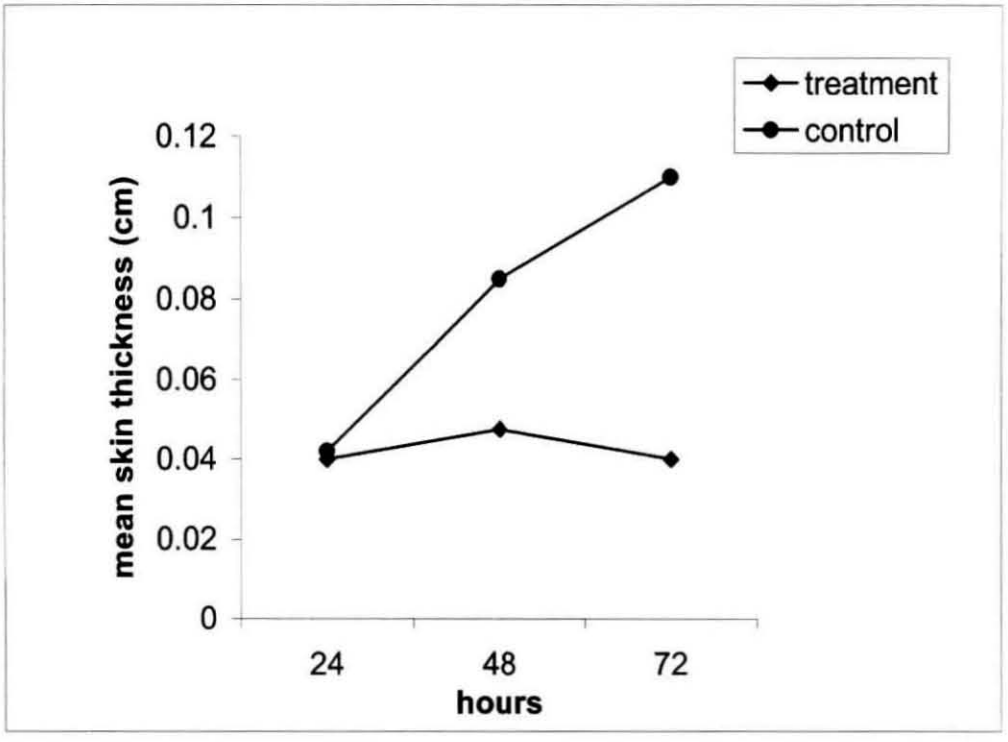


Figure 2. Effect of aflatoxin on the PHA response in *E. suratensis*

hepatic chords. The sinusoids joined the hepatic veins and biliary canaliculi joined to form larger bile ductules and ducts.

Initially in the treatment groups, the hepatocytes revealed degeneration. Many hepatocytes appeared vacuolated. Focal areas showed loss of hepatocytes and hepatocytes undergoing coagulative necrosis. Hepatocytes with shrunken nuclei were evident. Biliary epithelial proliferation and fibroblastic proliferation were noticed in parenchyma of liver and the stromal tissue became prominent. There was accumulation of mononuclear leucocytes around proliferating biliary ducts. The pancreatic islands showed degeneration and infiltration with leukocytes as well as necrosis. The tissues collected after four and six weeks indicated massive proliferation of biliary epithelium leading to formation of new biliary ductules and ducts along with fibrous tissue growth. Degeneration and necrosis were evident in many areas. There were areas of hepatic cell regeneration, which were indicated by hepatocytes with abundant basophilic cytoplasm and vesicular nuclei. (Plates 7a – 7f)

In livers collected from three fishes, which were fed with aflatoxin containing feed for eight weeks, there were black spots spread all over the surface of liver. These livers microscopically revealed proliferating hepatocytes, which formed basophilic foci of large plum cells in hepatic parenchyma. A large number of plum hepatocytes with different shapes were also noticed. Some of the cells had marked enlargement of cytoplasm and nuclei. Cells with more than one nuclei were also present. The liver parenchyma showed extensive degeneration and necrosis of the normal hepatocytes due to the massive proliferation of pleomorphic basophilic cells. These cells invaded the hepatic parenchyma. Some of them were with polyhedral shape and they had prominent nucleus with nucleoli. These cells were arranged in loose collections. There were large number of mitotic cells and focal basophilic megalocytes. In some places they were highly pleomorphic and formed no specific pattern indicating a carcinoma. This growth of new cells extensively invaded the parenchyma of the liver while the normal parenchyma in many places appeared necrotic and undergoing degenerative changes. There was extensive infiltration of leukocytes in the parenchyma of the liver. In other animals of this group, which had pale raised irregular patches on the liver surface, hyperplasia of hepatocytes was noticed. A large number of plum hepatocytes with different shapes was also noticed.

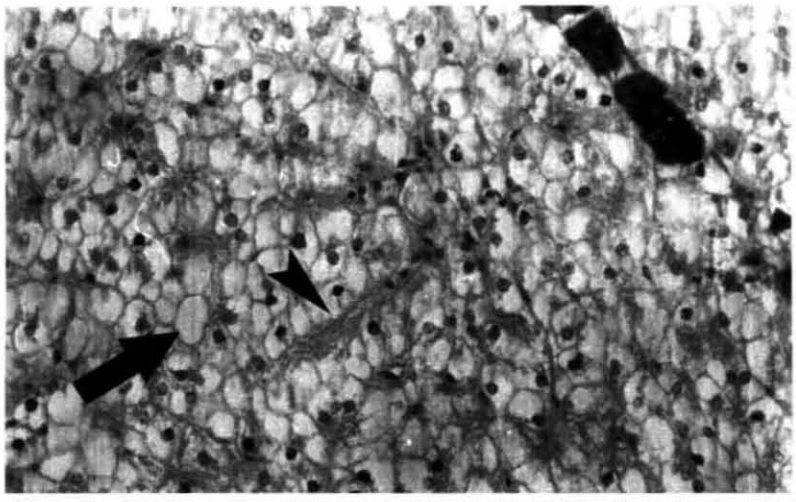


Plate 7a. Liver section of *E.suratensis* treated with aflatoxin showing vacoulation(arrow) and proliferation of fibrous tissue(arrow head). H&E.400x

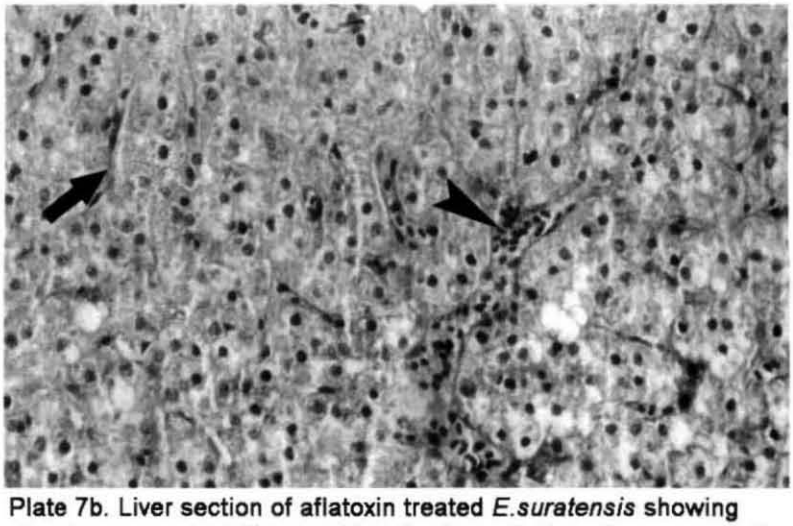


Plate 7b. Liver section of aflatoxin treated *E.suratensis* showing showing moderate biliary proliferation(arrow). Note the accumulation of leucocytes around proliferating new biliary tubules(arrow head). H&E. 400x.

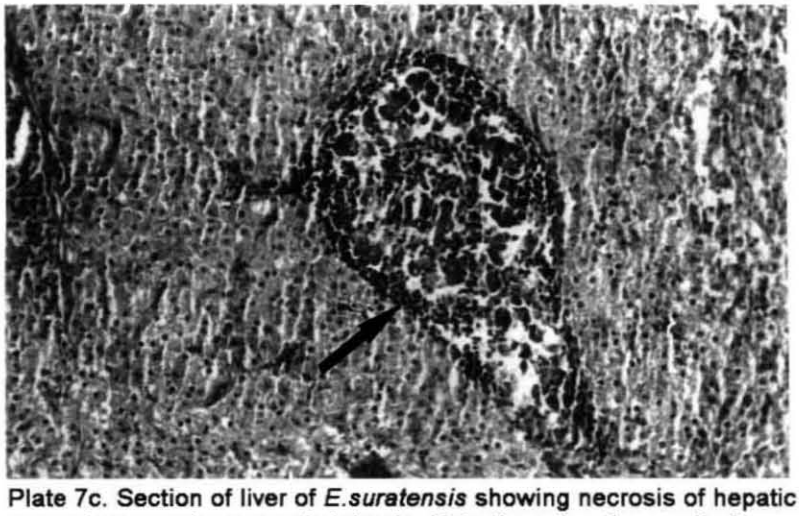


Plate 7c. Section of liver of *E.suratensis* showing necrosis of hepatic parenchyma and pancreatic islands. Note the pyknotic nuceli of hepatocytes & infiltration of leucocytes into the pancrearic acinar tissue. H & E 200x

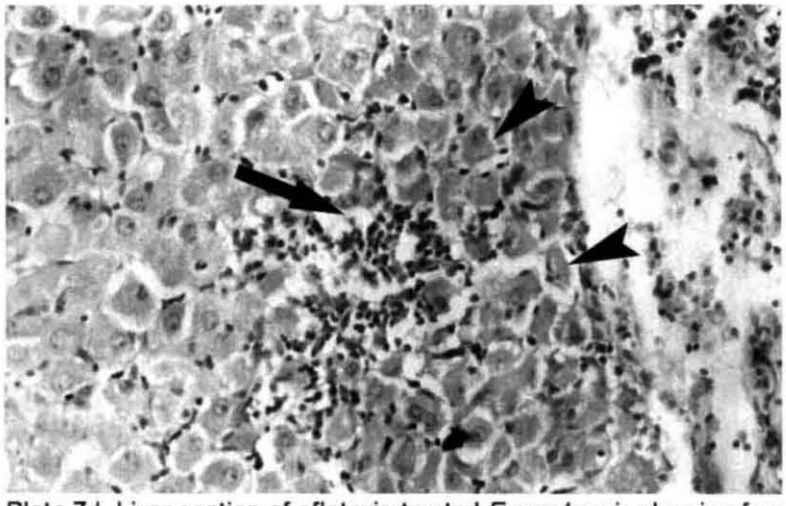


Plate 7d. Liver section of aflatoxin treated *E.suratensis* showing focal necrotic area infiltrated with leucocytes(arrow).Note also the polyhedral plum hepatocytes(arrow head) and mild biliary proliferation. H&E.400x

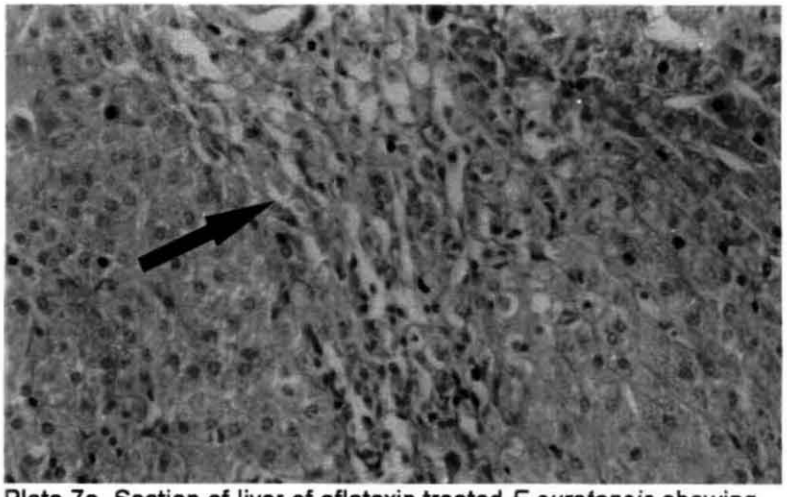


Plate 7e. Section of liver of aflatoxin treated *E.suratensis* showing extensive proliferation of biliary tissue in the parenchyma of liver. H&E. 400x

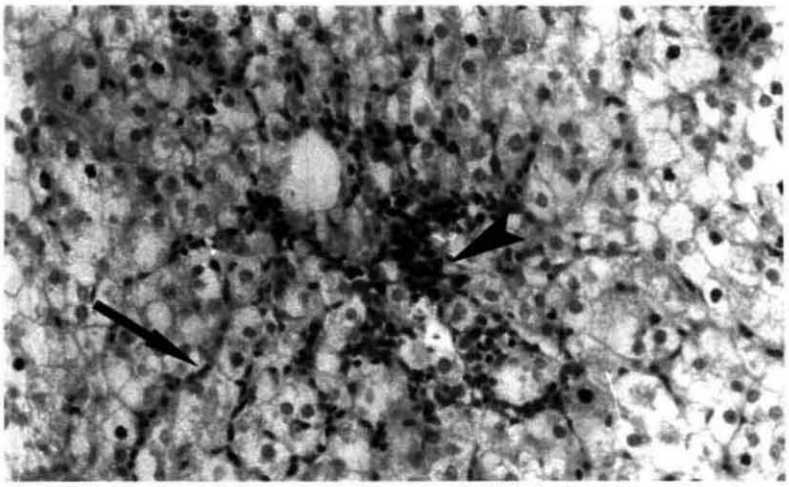


Plate 7f. Section of liver of aflatoxin treated fish showing extensive proliferation of biliary tissue(arrow) which is surrounded by infiltrating mononuclear cells(arrow head).Note the hepatocytes showing vacoulation. H&E. 400x.

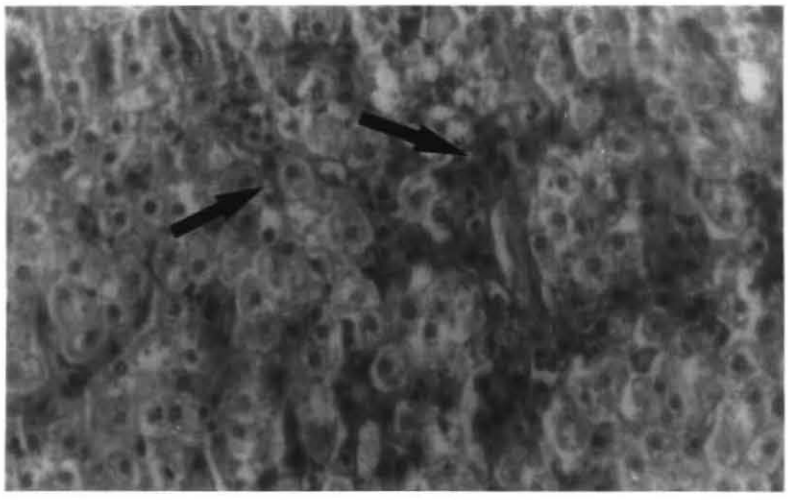


Plate 8a. Section of liver of aflatoxin treated *E.suratensis* revealing massive proliferation of biliary tissue. H&E. 400x

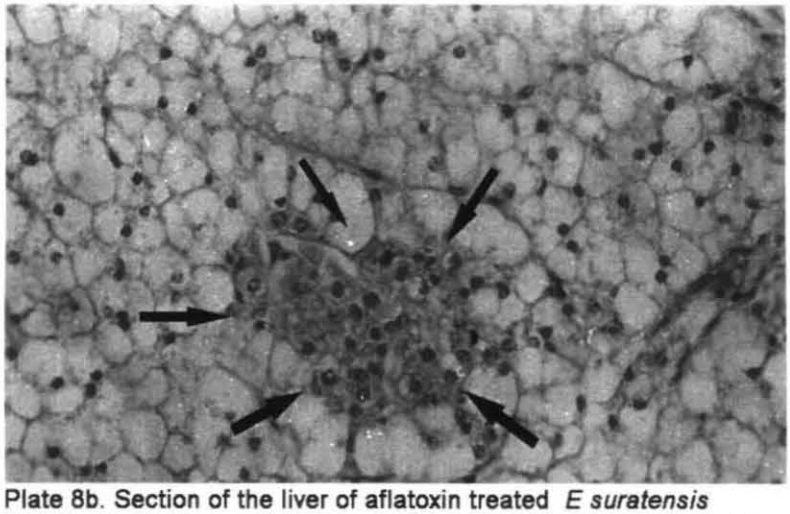


Plate 8b. Section of the liver of aflatoxin treated *E suratensis* showing foci of basophilic regenerating cells. Note the other cells showing vacoulation H&E. 400x

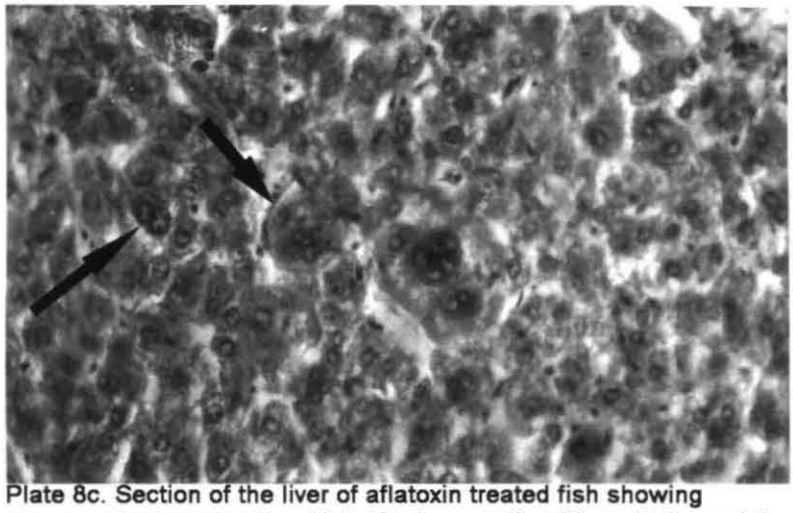


Plate 8c. Section of the liver of aflatoxin treated fish showing regenerating hepatocytes. Note the large cells with multiple nuclei and also other cells with more than one nuclei. H&E.400x

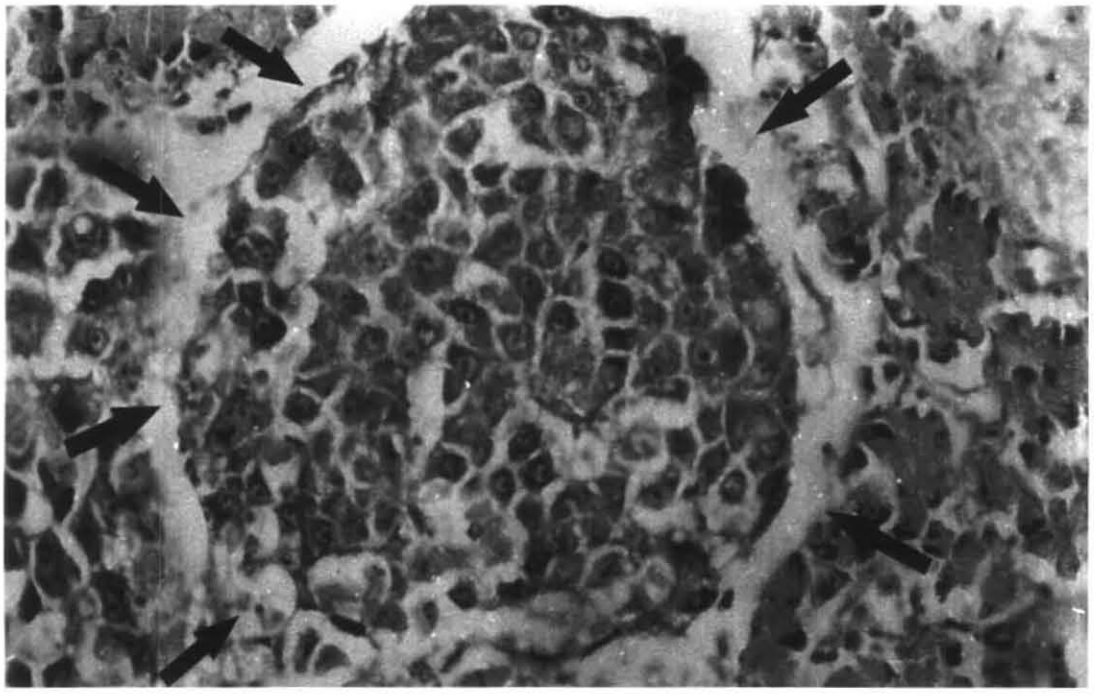


Plate 8d. Section of the liver of aflatoxin treated *E.suratensis* for 8 weeks showing proliferation of anaplastic pleomorphic cells indicating induction of carcinoma. H&E. 400x

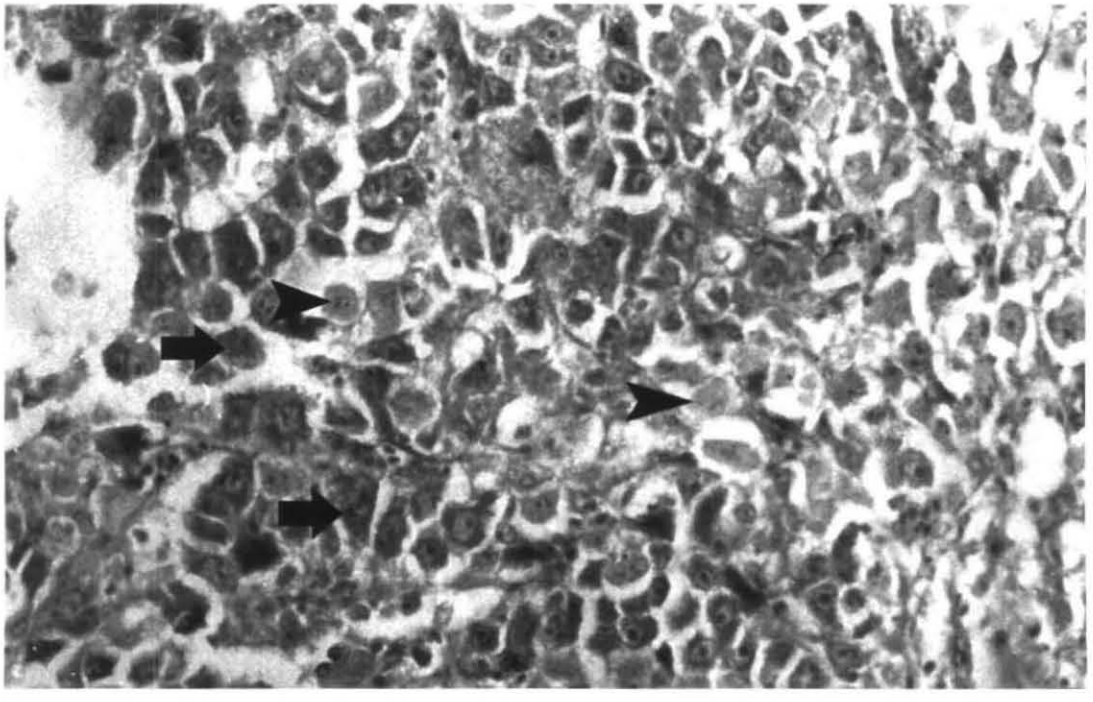


Plate 8e. Liver section from *E.suratensis* fed aflatoxin for 8 weeks showing growth of pleomorphic polyhedral anaplastic cells(arrow). Note also the normal hepatocytes in between the growing neoplastic cells(arrow head). H&E 400x

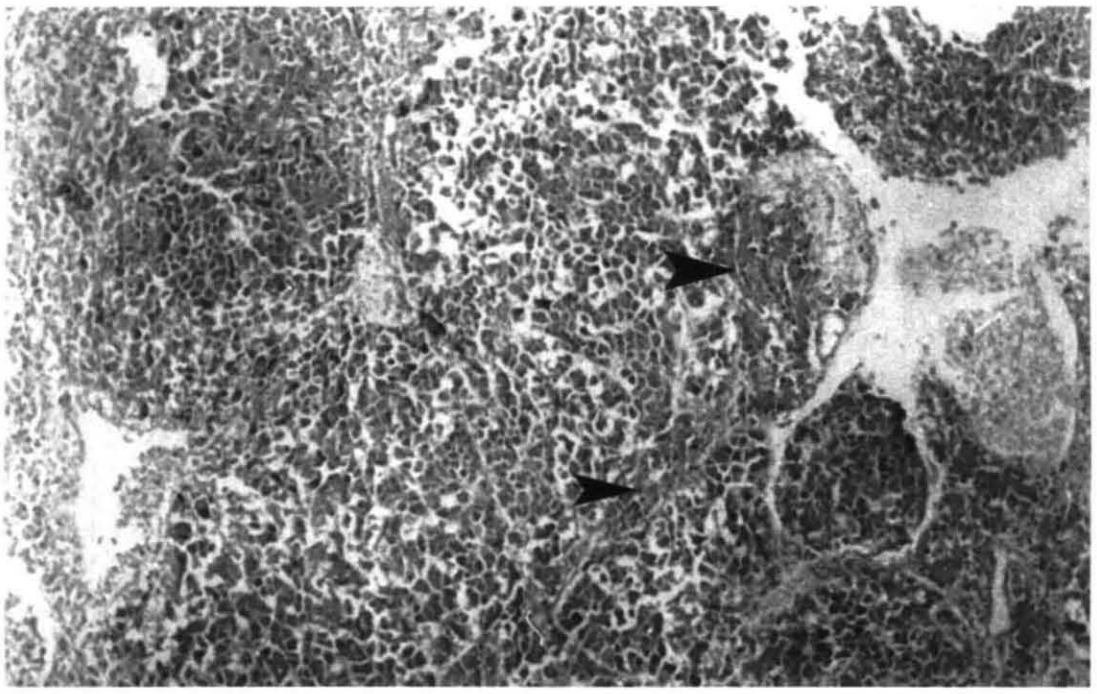


Plate 8f. Section of liver from aflatoxin treated *E. suratensis* for 8 weeks showing massive growth of pleomorphic anaplastic cells into the liver parenchyma replacing most of the normal hepatocytes. Only islands of normal hepatocytes are seen(arrow head).H&E 400x

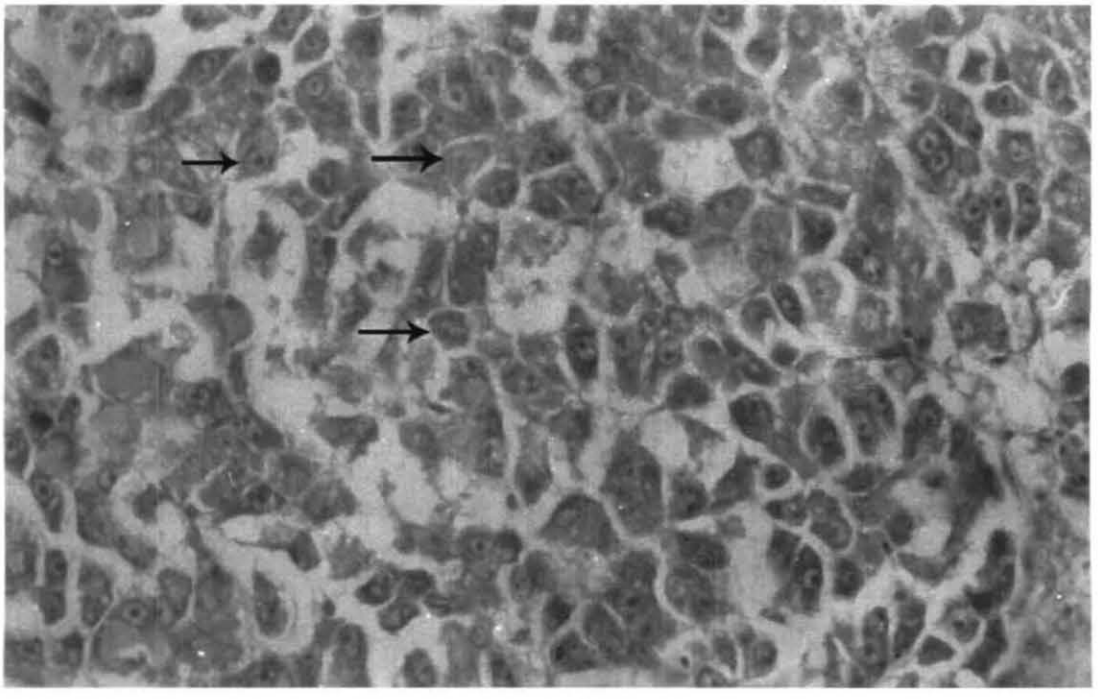


Plate 8g. Section of liver from *E.suratensis* treated with aflatoxin for 8 weeks. Note the polyhedral, pleomorphic neoplastic cells. H&E 400x

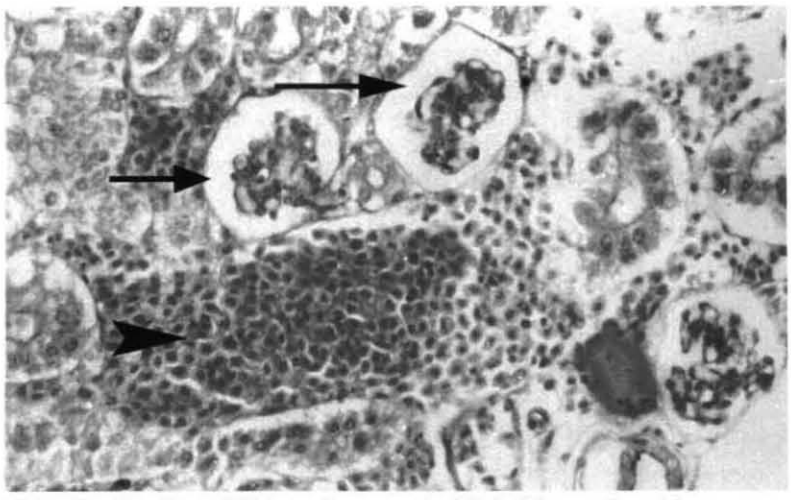


Plate 9a. Section of kidney from control fish (*E. suratensis*) showing tubular structures, glomeruli(arrow) and haemopoietic area(arrow head).H&E 400x.

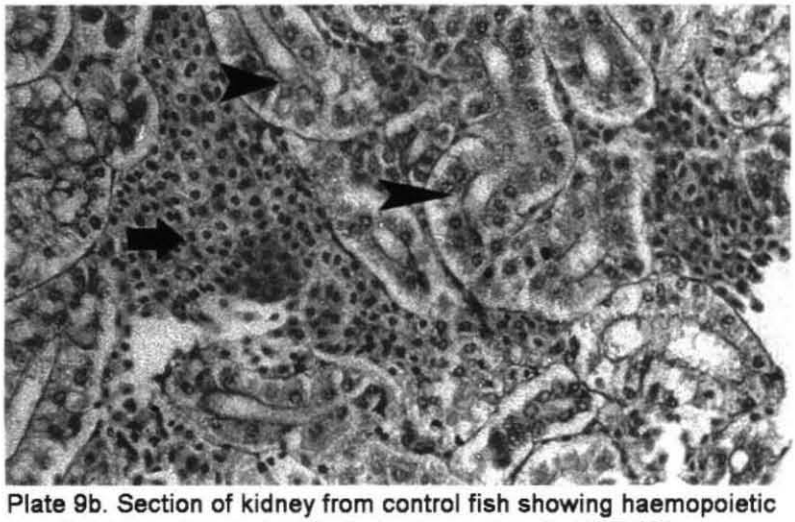


Plate 9b. Section of kidney from control fish showing haemopoietic area(arrow) and convoluted tubules(arrow head). H&E 400x

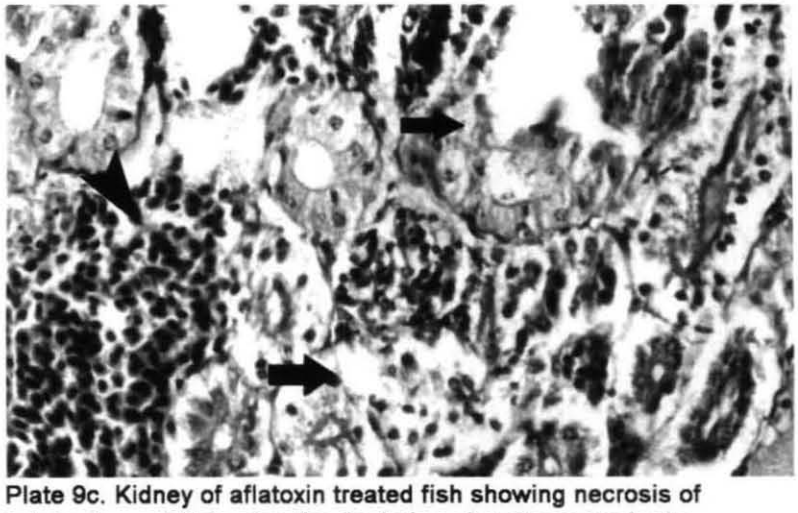


Plate 9c. Kidney of aflatoxin treated fish showing necrosis of tubules(arrow).Note also the depletion of erythrocytes in the haemopoietic area(arrow head). H&E 400x

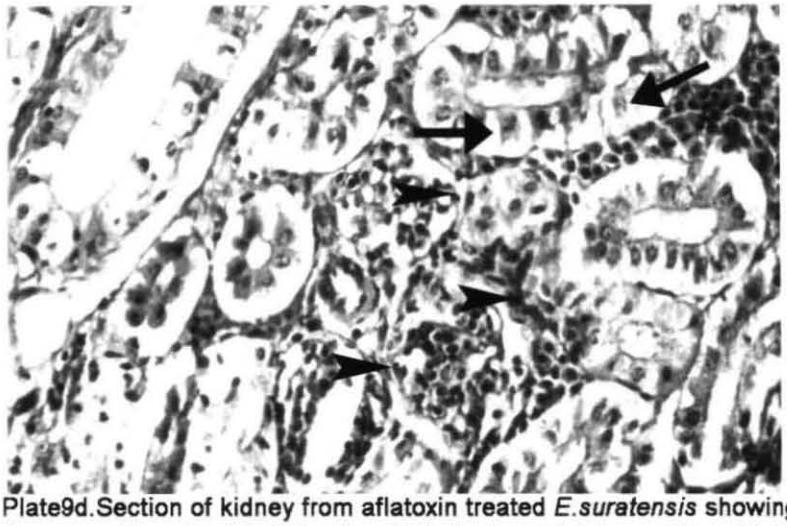


Plate9d.Section of kidney from aflatoxin treated *E.suratensis* showing desquamation of epithelial cells of tubules(arrow).Intercapillary thickening of glomerular capillaries, proliferation of parietal layer of bowman's capsule and adhesion of glomeruli with parietal layer of bowman's capsule is also seen(arrow head). H&E 400x

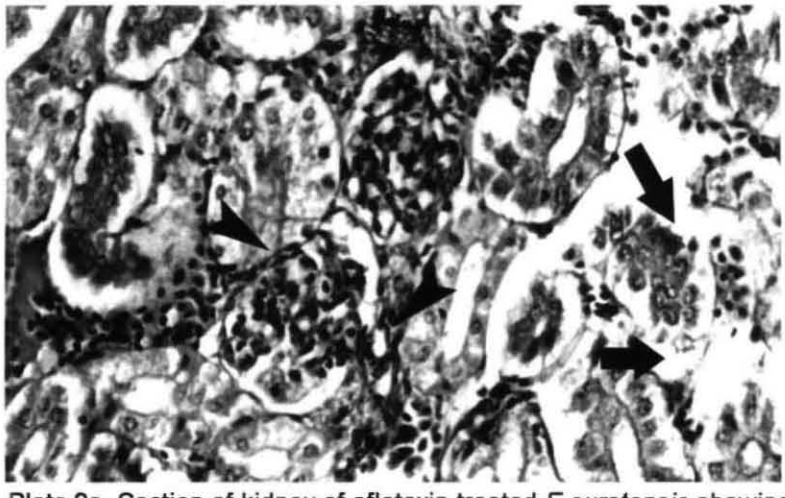


Plate 9e. Section of kidney of aflatoxin treated *E suratensis* showing necrosis of tubular epithelium(arrow), periglomerular fibrosis and adhesion of glomeruli and intercapillary thickening(arrow head). H&E 400x

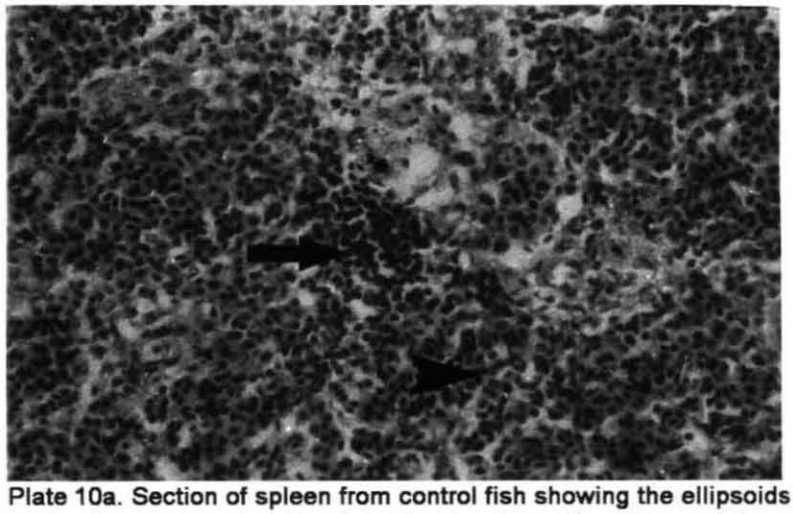


Plate 10a. Section of spleen from control fish showing the ellipsoids containing phagocytic cells, the red pulp with erythrocytes and the lymphoid tissue containing large number of lymphoblasts. H&E 400x

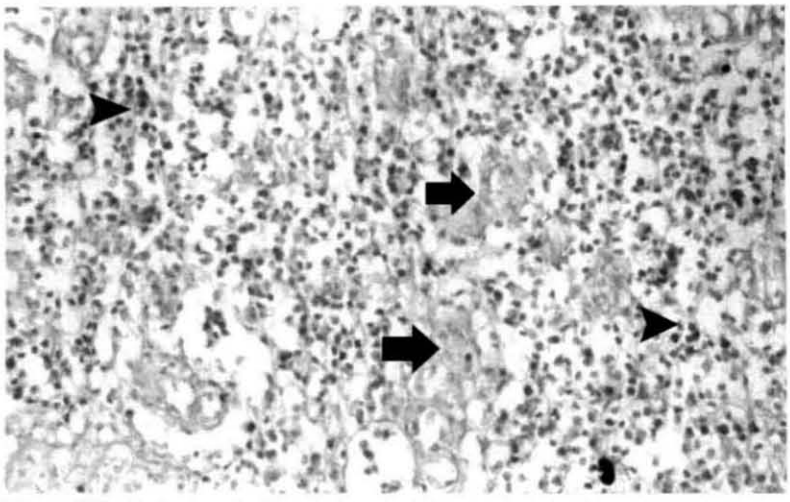


Plate 10b. Spleen of afltoxin treated *E.suratensis*. Note the depletion of cells from ellipsoids(arrow) and the pyknotic nuceli of lymphoid tissue(arrow head).H&E 400x

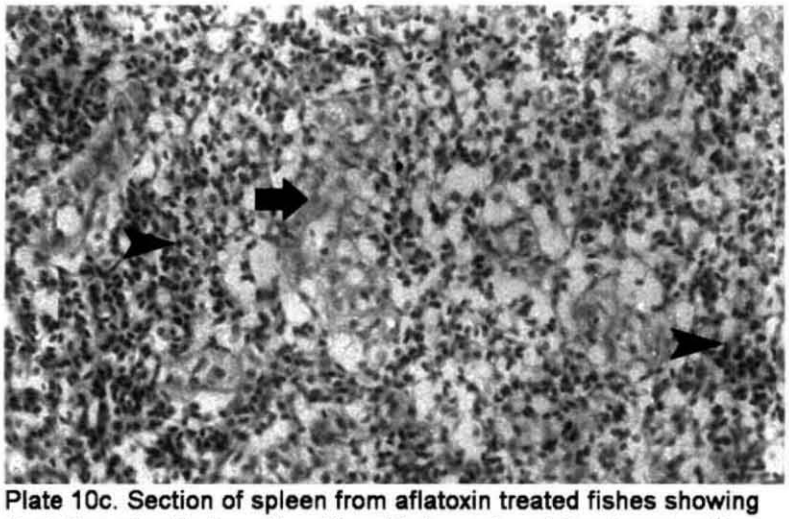


Plate 10c. Section of spleen from aflatoxin treated fishes showing depletion of cells from the ellipsoids(arrow) and the degenerative changes in lymphoid tissue indicated by cells with pyknotic nuclei(arrow head).H&E 400x

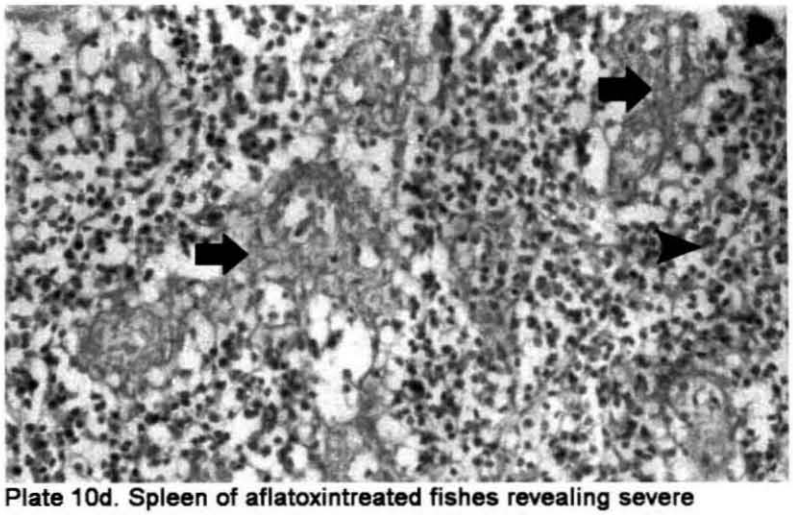


Plate 10d. Spleen of aflatoxintreated fishes revealing severe degenerative changes in the lymphoid tissue(arrow head) and ellipsoids(arrow). H&E 400x

4.1.5.4. Thymus

In control fish group, the thymus had diffused lymphoid tissue interspersed with occasional epithelial cells. (Plates 11a & 11b)

In aflatoxin treated fishes, the lymphoid tissue had areas of haemorrhages and the lymhocytes were destroyed. In some cases, thymus revealed depletion of lymhocytes in the parenchyma.(Plate 11c)

4.1.6. Ultrastructural Studies

Liver, kidney, spleen and thymus were examined for assessing the ultrastructural alterations brought about by aflatoxin.

4.1.6.1. Liver

In the control fishes, hepatocytes had a rich profile of rough and smooth endoplasmic reticulum (ER) and the smooth ER occupied the peripheral area of the cytoplasm. In between rough ER and smooth ER, round and rod shaped mitochondria with numerous cristae and granules were seen. The hepatocytes had well developed stack of golgi apparatus. The surface of the hepatocytes lining the sinusoid and biliary canaliculi had numerous fingerlike evaginations (microvilli) projecting into the space of Disse. The endothelial cells lining the sinusoids had irregular nucleus. The nucleus of the hepatocytes appeared spherical with abundant euchromatin and well developed nucleolus. Cytoplasm of hepatocytes had numerous free ribosomes scattered in the matrix. (Plate 12a – 12d)

In aflatoxin treated fishes, progressive changes were noticed over the 8-week exposure period. Initially the nucleus became irregular in shape. The nucleus contained electron dense inclusions, which may be probably protein inclusions. The amount of heterochromatin in the nucleus increased. The nucleus also contained chromatin granules and perichromatin granules. The mitochondria lost its structure. Many of them appeared swollen, with disappearing cristae and granules. There was vacuolation in the cytoplasm. In many cells, there was proliferation of ER forming large whirls in the cytoplasm. Numerous electron luscent spherical structures with

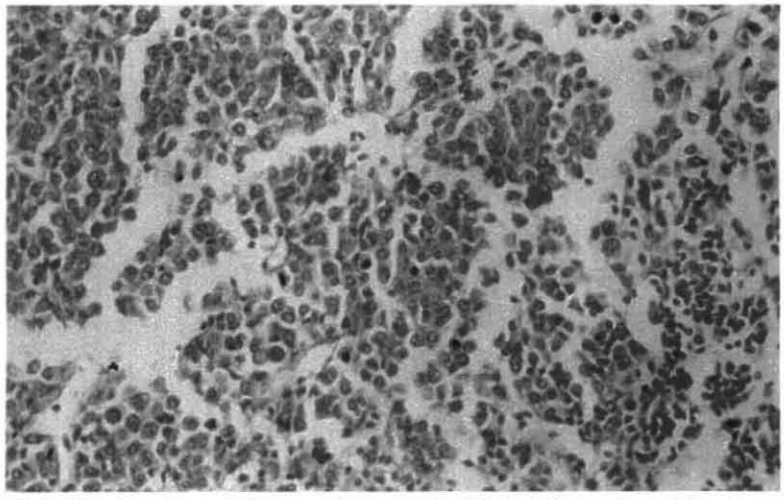


Plate11a. Section of thymus from control fish (*E.suratensis*) showing proliferating lymphoblasts in the parenchyma. H&E. 400x

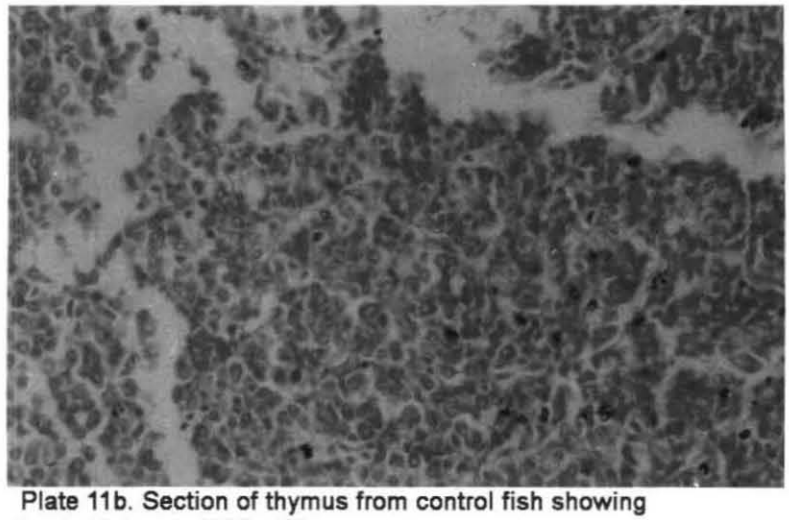


Plate 11b. Section of thymus from control fish showing lymhoid tissue. H&E .400x

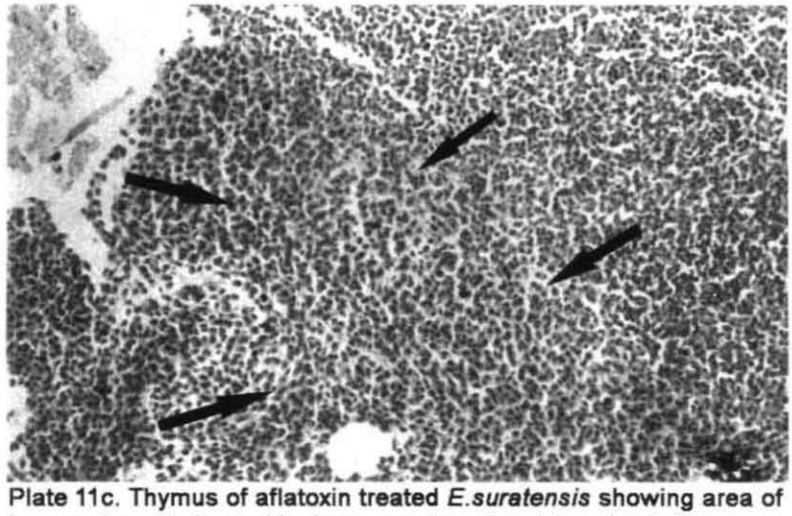


Plate 11c. Thymus of aflatoxin treated *E.suratensis* showing area of haemorrhage indicated by large number of eryhtrocytes in the area H&E. 200x

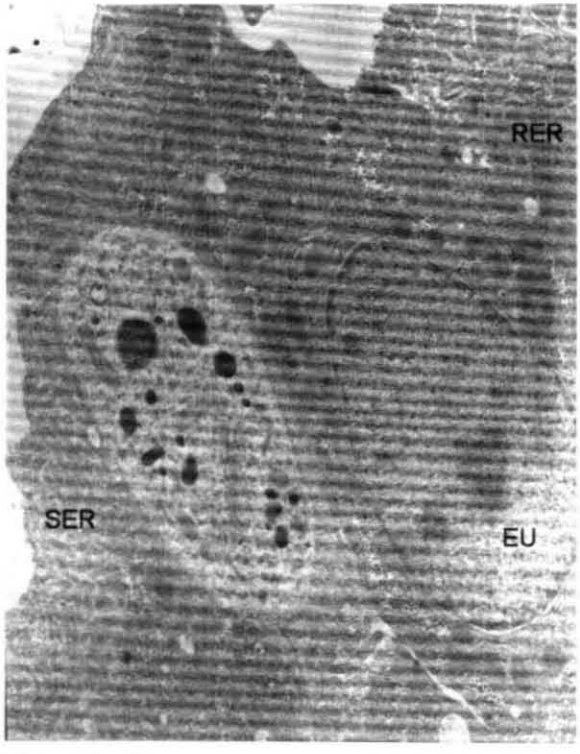


Plate 12a. Electron micrograph of the hepatocyte of control fish (*E.suratensis)* showing extensive smooth and rough ER(SER,RER), mitochondria and nuclei with abundant euchromatin(EU). 8000x

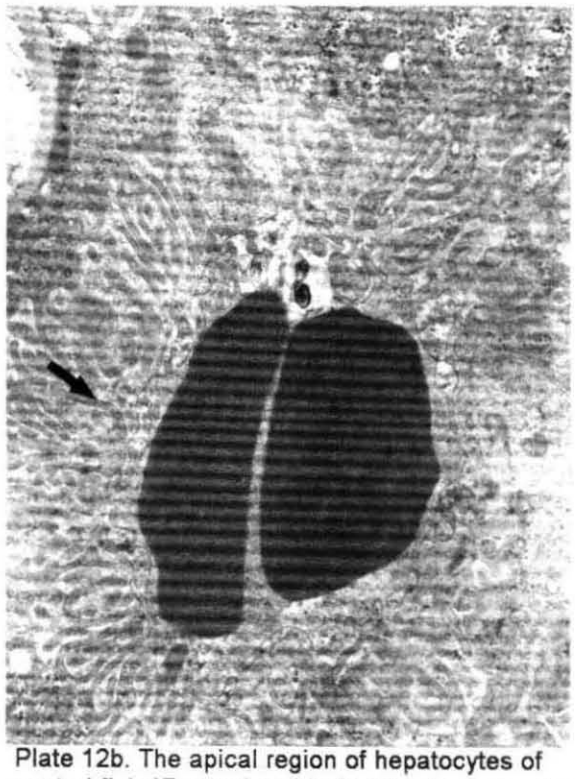


Plate 12b. The apical region of hepatocytes of control fish (*E. suratensis*) showing large number of microvilli projecting into the space of Disse. 5000x

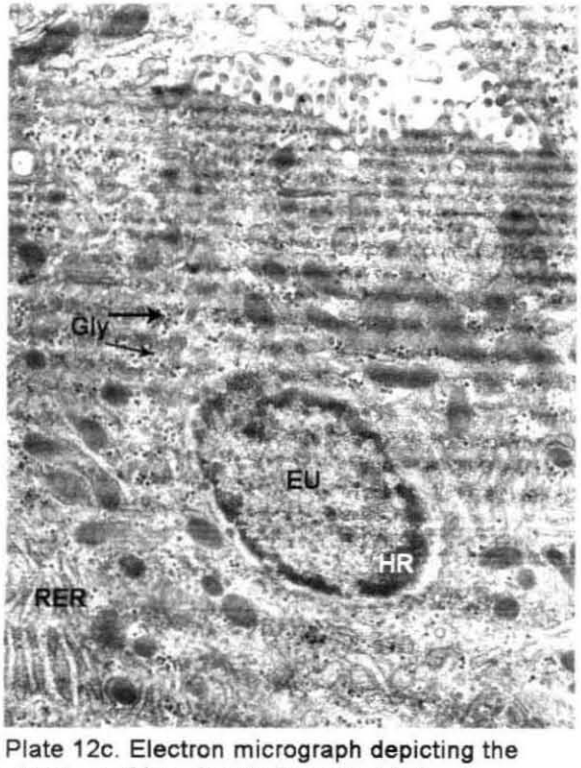


Plate 12c. Electron micrograph depicting the structure of hepatocyte in control fish (*E. suratensis*). Note the abundant RER, large number of mitochondria, presence of glycogen deposits and nucleus having abundant euchromatin (EU)and moderate amount of heterochromatin(HR) adhering to the nucelar membrane. 5000x

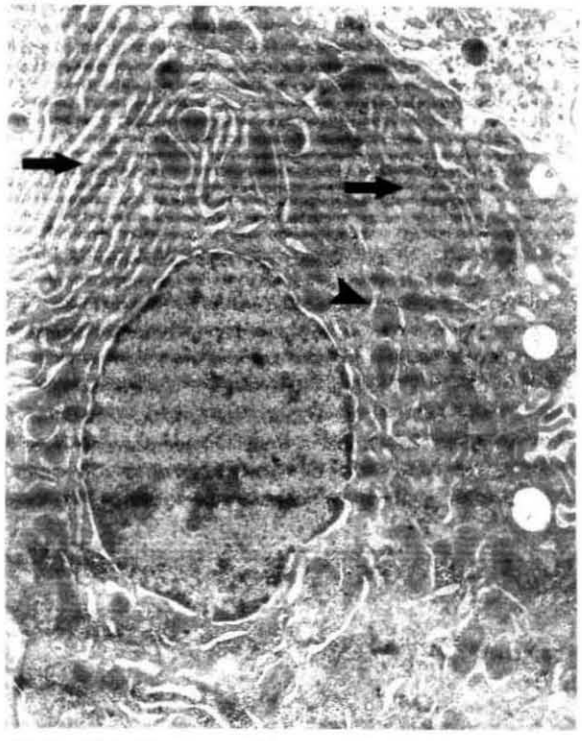


Plate 12d. Electron micrograph of hepatocyte from control fish (*E. suratensis*) showing extensive rough ER(arrow) and mitochondria(arrow head). 4000x

dense core appeared in the cytoplasm indicating production of peroxisomes. The rough ER lost ribosomes and this was followed by fragmentation of SER and RER. Large membrane bound vesicles containing electron luscent materials were seen in the cytoplasm. Numerous liposomes also appeared in the cytoplasm. The ER in many cells had undergone dilatation and formation of large vesicles was also observed. The microvilli of hepatocytes lost its integrity, the desmosomes were destroyed and the plasmalemma as well as nuclear membrane lost its continuity. In many cells multivesicular bodies and autophagosomes containing condensed organelles at different stages of destruction could be seen. (Plates 12e – 12l)

4.1.6.2. Kidney

In the control group kidney had haemopoietic tissue which was traversed with numerous sinuses lined by loose endothelial cells. The lumen contained different type of cells. These cells had nuclei with abundant euchromatin and small clumps of heterochromatin. The cells were having numerous polyribosomes in their cytoplasm. The periphery of the sinuses was occupied by cells with membrane bound granules and also cells without granules but with large nuclei. Some of the cells with granules had nucleus with more heterochromatin. Granules were of two types, one with a crystalloid core surrounded by moderately electron dense matrix. The other type contained homogenous electron dense material. The excretory portion of the kidney was composed of glomeruli and tubules. The proximal convoluted epithelial cells had elongated appearance with numerous apical microvilli. Below the microvilli a thick coat of glycocalyx could be seen. The cells showed basal infoldings and rich profile of RER. Numerous rod shaped mitochondria could be seen in the basal part of the cell. The distal convoluted tubule showed cells with numerous basal tubular infoldings which were continuous with the RER. Numerous mitochondria were seen in between the foldings. The nucleus of the cells had prominent nucleolus. The glomeruli were formed of capillaries lined by fenestrated endothelial cells with electron luscent basement membrane support. The fenestrations of capillaries were partially covered by fingerlike processes of epithelial cells forming the visceral layer of Bowman's capsule. (Plate 13a – 13d)

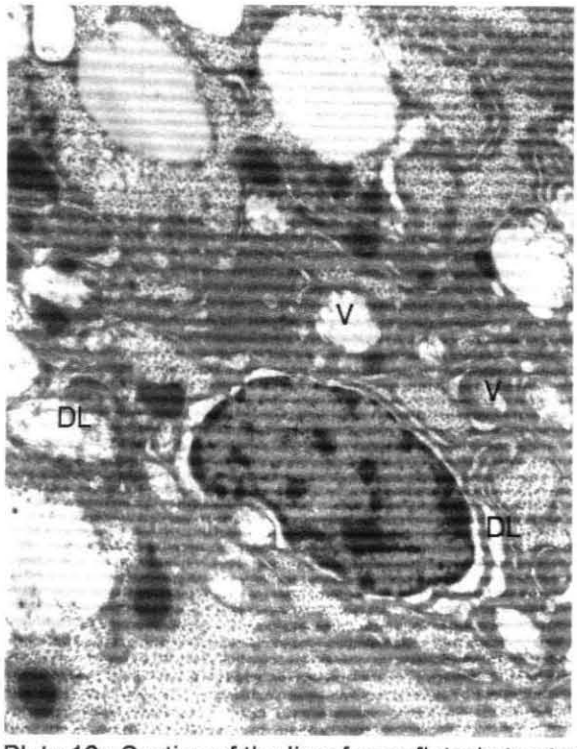


Plate 12e.Section of the liver from aflatoxin treated *E.suratensis*. Note the dilatation(DL) of lumen of theER, vesiculation(V) and condensation of mitochondria. Degranulation of ER and compression of the nucleus is also seen.10,000x

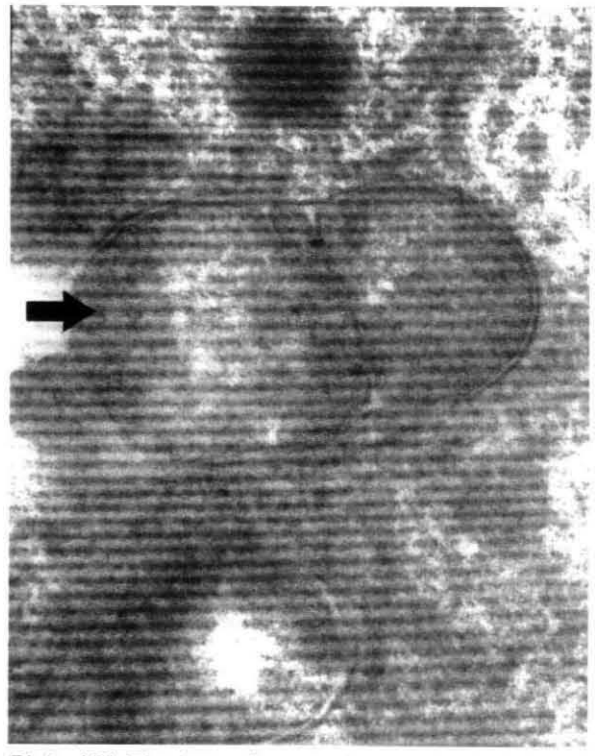


Plate 12f. Electron micrograph of the liver of aflatoxin treated *E.suratensis* showing swollen mitochondria which is devoid of cristae and granules and is surrounded by ER forming autophagic vesicles 80,000x

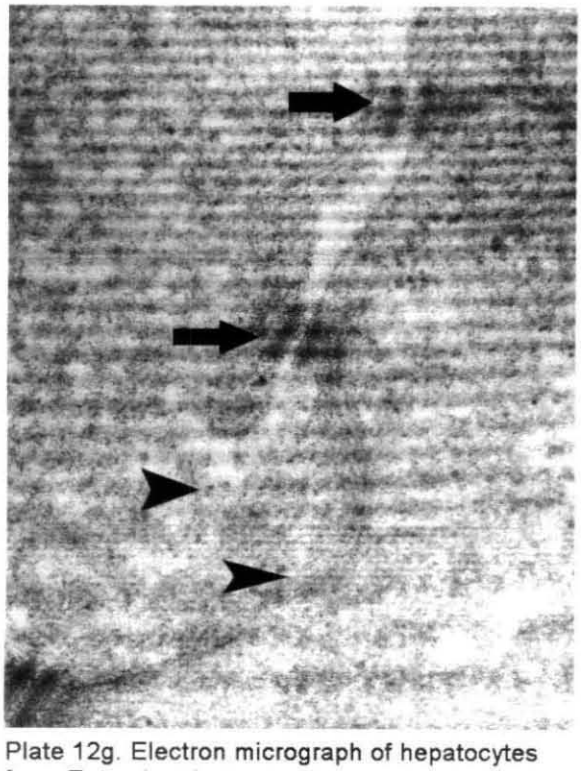


Plate 12g. Electron micrograph of hepatocytes from *E.suratensis* exposed to aflatoxin showing the loss of contact between cells indicated by the separation of desmosomes(arrow) and loss of integrity of cell membranes(arrow head).The disassembly ofmicrotubules is also evident.60,000x

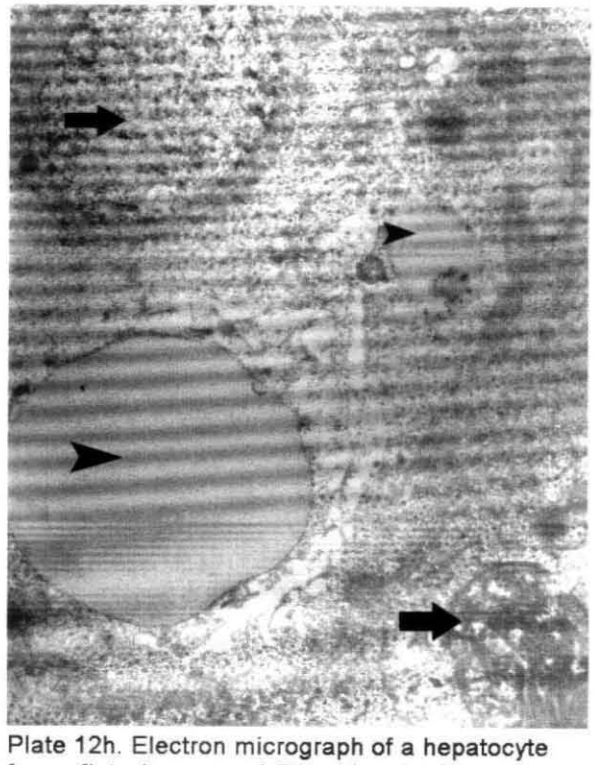


Plate 12h. Electron micrograph of a hepatocyte from aflatoxin exposed *E.suratensis* . Note the presence of autophagic vesicle(arrow) and electron luscent material(arrow head) in the cytoplasm.10,000x

Plate 12i. Hepatocyte from aflatoxin exposed *E.suratensis* showing autophagic vesicle with different organelles in it. 25,000x

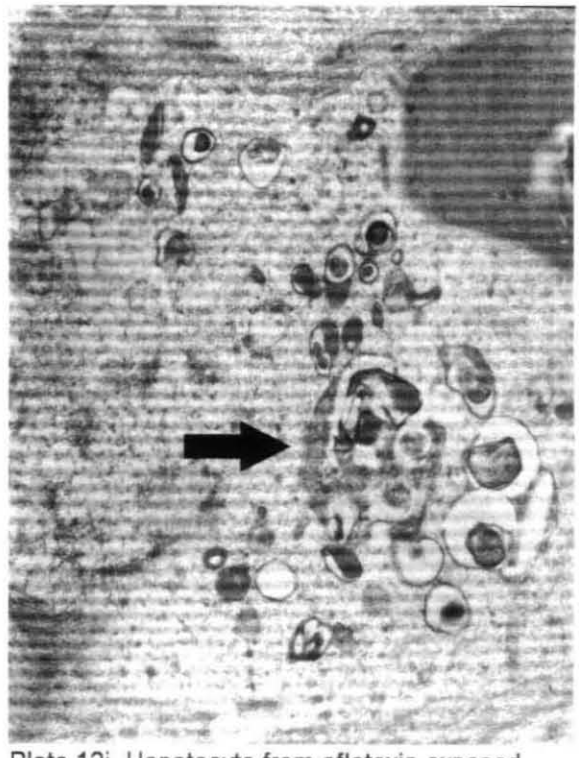


Plate 12j. Hepatocyte from aflatoxin exposed *E.suratensis* depicting different stages of autophagic vacoules, multi vesicular bodies and formation of lipofuchsin. 10,000x

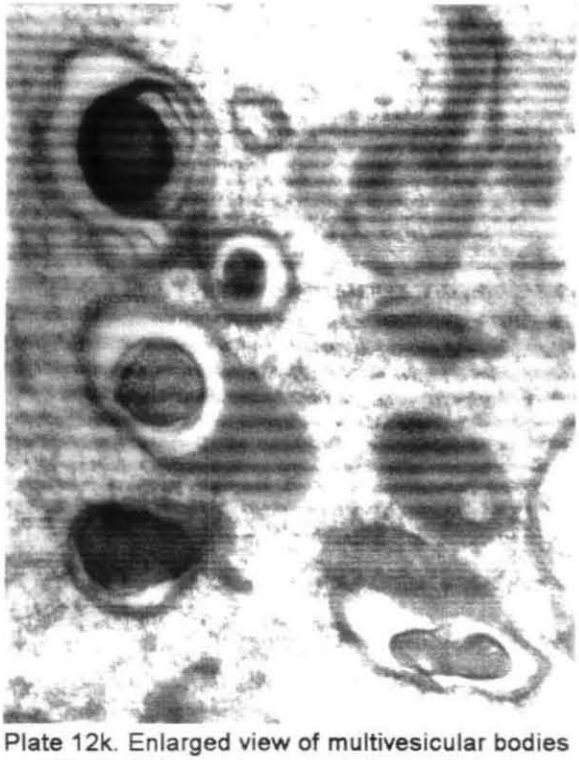


Plate 12k. Enlarged view of multivesicular bodies and lipofuchsin in a hepatocyte from aflatoxin treated *E.suratensis*. 50,000x

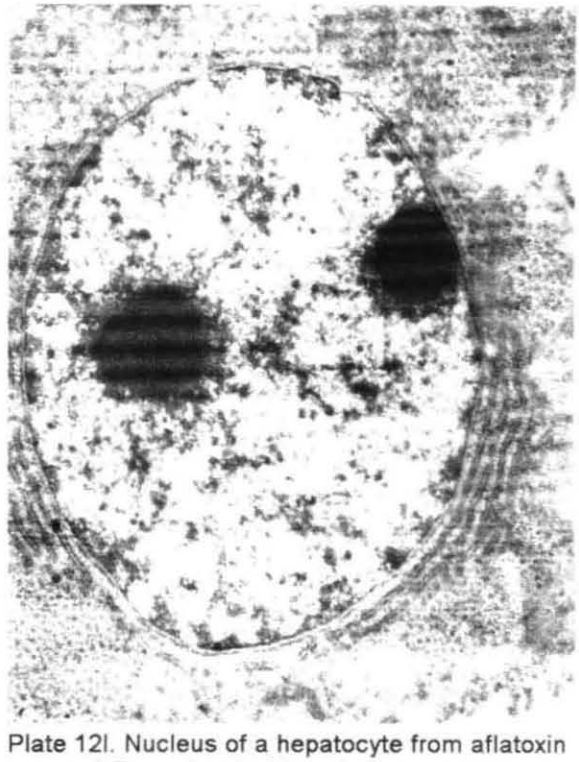


Plate 12l. Nucleus of a hepatocyte from aflatoxin exposed *E.suratensis*. Note the presence of electron dense inclusions and loss of chromatin material; and the fragmentation and loss of ribosomes from ER at the periphery of nucleus. Free ribosomes in the cytoplasmic matrix is also seen. 15,000x

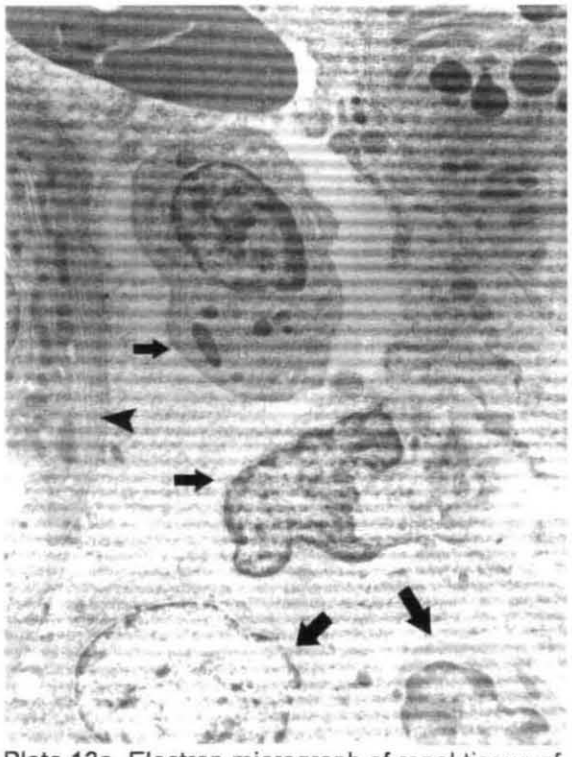


Plate 13a. Electron micrograph of renal tissue of control fish (*E. suratensis*) showing the interstitial haemopoietic area. Note the developing granulocytes and lymphocytes(arrow) alongwith adjacent epithelial cells(arrow head). 6000x

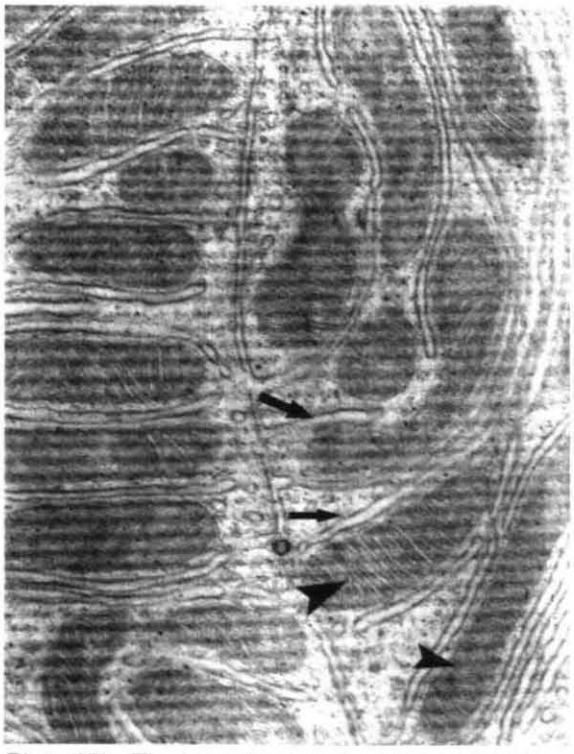


Plate 13b. Electronmicrograph of kidney tubular epithelial cells of control fish(*E. suratensis*) with basal infoldings of cell membranes(arrow)and large number of mitochondria(arrow head) in between. 30,000x

Plate 13c. Electronmicrograph of the kidney of control fish(*E. suratensis*) showing glomerular structre. Note the foot processes of the podocytes in close contact with the basement membrane layer of capillaries. 15,000x

Plate 13d. Kidney glomerular capillary structure of control fish (*E. suratensis*). The parietal podocyte processes are in close contact with basement membrane(arrow). Also note the presence of mesenchymal cells with elliptical nuclei (arrow head). 8000x

Appearance of vesicles and increase in heterochromatin clumps in the nucleus were observed in the the haemopoietic cells of aflatoxin treated fishes. Mitochondria revealed condensation, lysosomes contained electron dense amorphous material. In the epithelial cells the microvilli were lost and dilatation of ER was also observed in tissues collected during the initial four weeks of aflatoxin exposure. Desmosomes got separated, RER lost ribosomes and dilated forming large vesicles. Mitochondria became swollen, contained amorphous material, inner matrix was condensed and granules as well as cristae were lost. ER in many cells showed fragmentation. Condensation and peroxidation of ER resulting in conformational changes and accumulation of densities were evident in kidney of fishes exposed to aflatoxin for a longer duration. Autophagocytosis of mitochobdria and other organelles were also seen. (Plate 13e – 13l)

4.1.6.3. Spleen

The spleen of the control fishes revealed numerous blood sinuses lined with loose endothelial cells. The sinuses contained cells with large nuclei containing abundant euchromatin. The cytoplasm was scanty and contained polyribosomes. Such cells were numerous in sinuses. Sinuses also contained cells with granules as described in the case of kidney. Developed erythrocytes were also seen in the sinuses. (Plate 14a – 14d)

In aflatoxin treated fishes the cells revealed peroxidation of ER membrane. ER fragmentation and severe mitochondrial damages like swelling, condensation of matrix and loss of cristae were also seen. The cell membranes were damaged and were discontinuous. Autophagy and nuclear changes like increase in heterochromatin and loss of integrity of nuclear membrane were also observed. (Plate 14e – 14l)

4.1.6.4. Thymus

The thymus structure in control fishes revealed large number of sinuses into which lymphoblasts were proliferating. There were a large number of interdigitating epithelial type of cells. These cells had large tubular interdigitating cytoplasmic processes, which were in close contact with the lymphoblasts. Some cells contained electron dense granules. In aflatoxin treated fishes, severe destruction of thymic parenchyma was seen (Plates 15a – 15d)

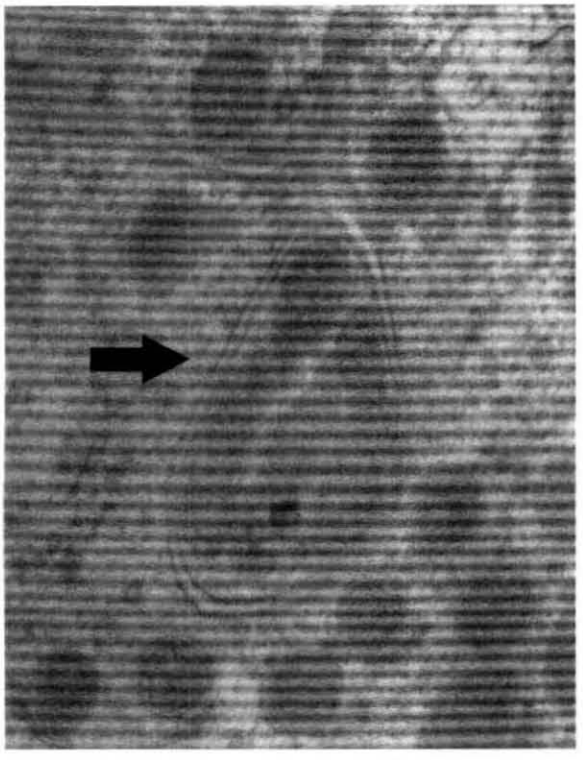


Plate 13e. Electron micrograph of a part of renal epithelial cell from aflatoxin treated *E.suratensis* depicting the degeneration and autophagy of mitochondria. 40,000x

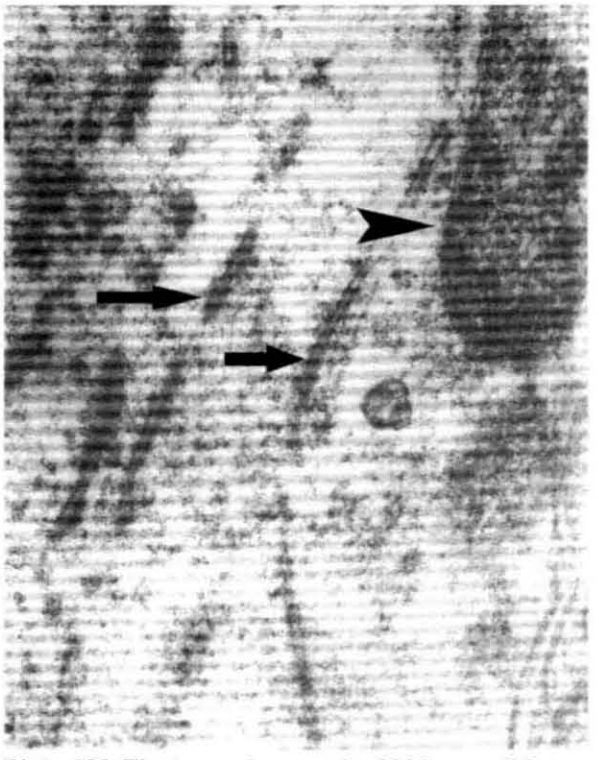


Plate 13f. Electron micrograph of kidney cell from aflatoxin treated *E.suratensis* revealing ER fragmentation(arrow) and loss of granules and cristae from mitochondria(arrow head). 60,000x

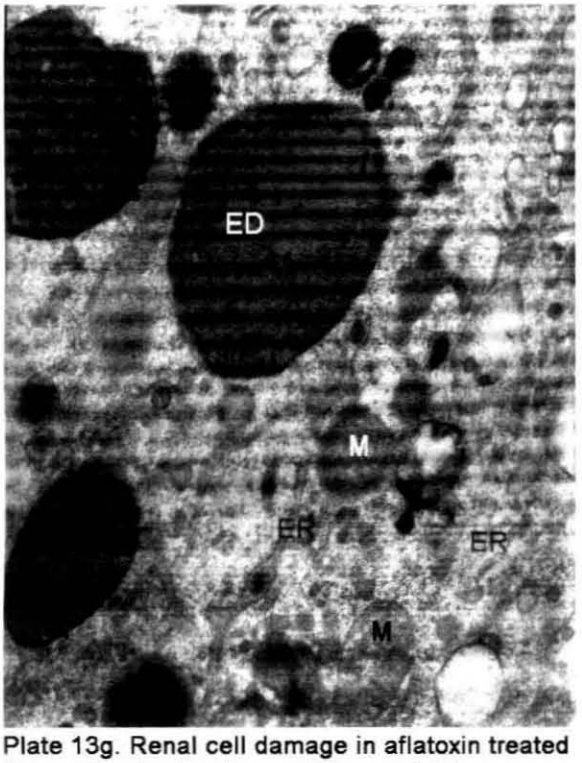


Plate 13g. Renal cell damage in aflatoxin treated *E. suratensis* showing vesicles containing electron dense(ED) materials. Also note the fragmented ER, condensation and loss of cristae from mitochondria(M). 20,000x

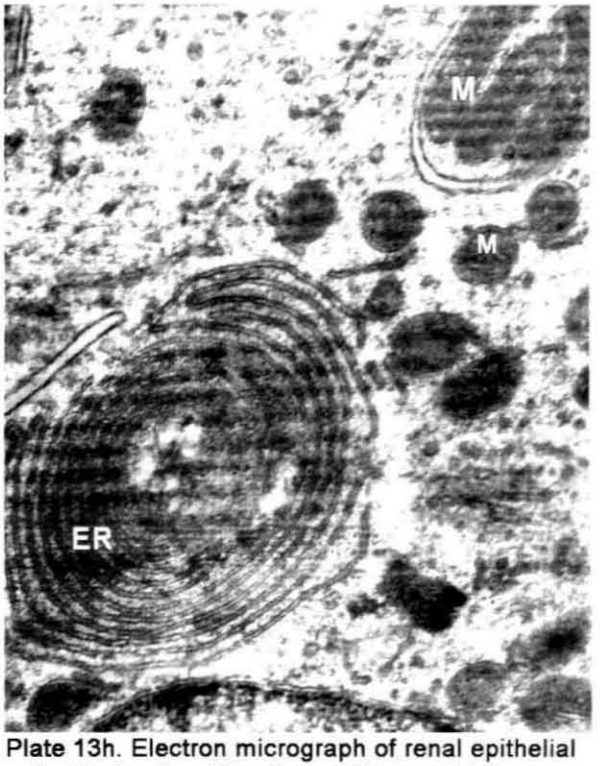


Plate 13h. Electron micrograph of renal epithelial cell with focal proliferation of ER in whirl form. Note the condensation and autophagocytosis of mitochondria(M). 30,000x

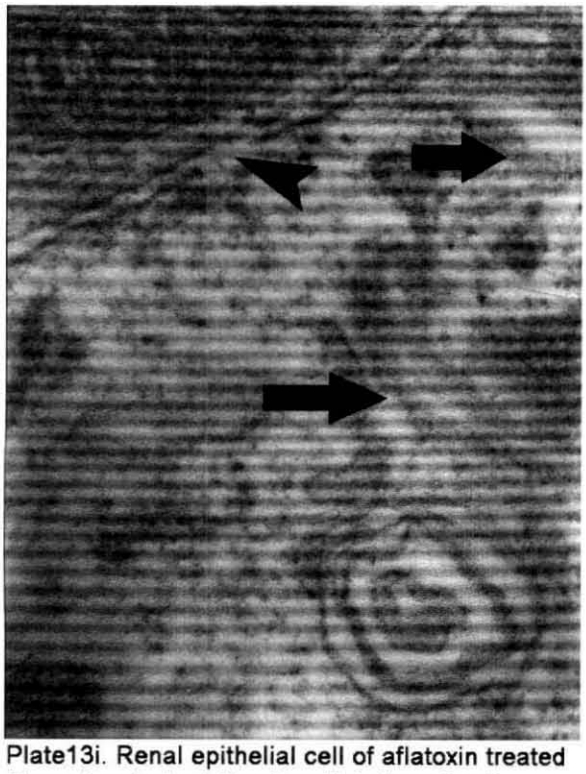


Plate13i. Renal epithelial cell of aflatoxin treated *E.suratensis* depicting the dilatation of cell membrane(arrow). Also note the breakage of plasmalemma(arrow head). 80,000x

Plate 13j. Electron micrograph showing the extensive mitochondrial damage in the kidney cells of *E.suratensis* treated with aflatoxin. 17,000x

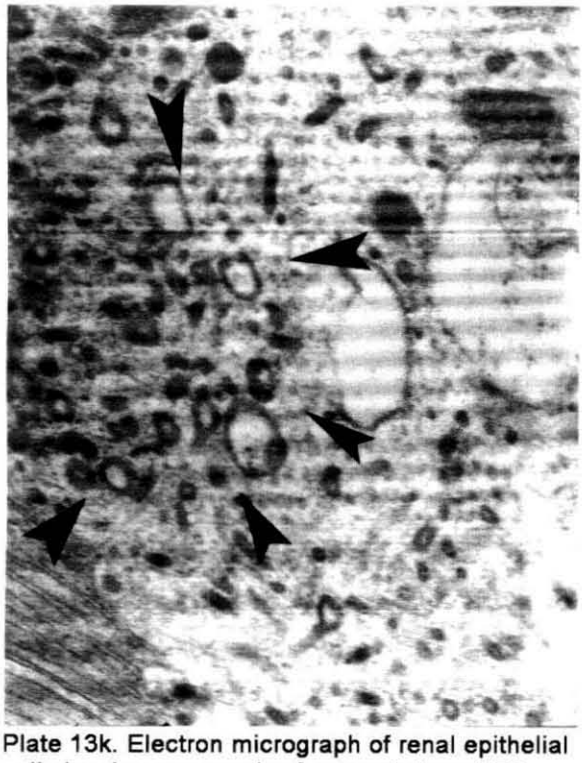


Plate 13k. Electron micrograph of renal epithelial cell showing progressive fragmentation of ER. Note the membranes showing accumulation of densities probably due to peroxidation. 30,000x

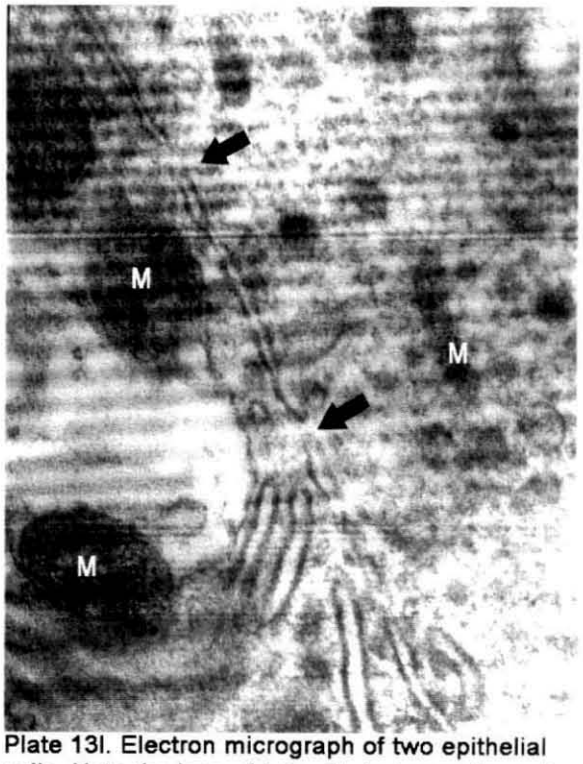


Plate 13l. Electron micrograph of two epithelial cells. Note the loss of integrity between the cell membranes(arrows), condensation of mitochondria(M) with granular electron dense material accumulating in the matrix and the fragmentation of ER.80,000x

Plate 14a. Section of spleen from control fish (*E. suratensis*) depicting the development of a granulocyte with a few granules in the cytoplasm and condensed nuclei. 8000x

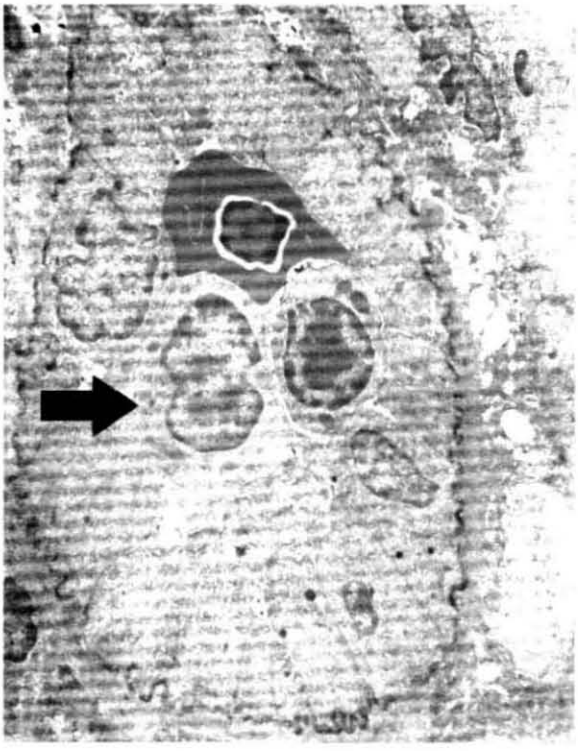


Plate 14b. Spleen cell of control fish (*E. suratensis*) showing a sinus. The multiplying lymphocyte seen in the lumen of the sinus.5000x

Plate 14c. Section of spleen from control fish depicting haemopoiesis. Note the dividing cells with appearance of chromosomes. 5000x

Plate 14d. Section of spleen from control fish (*E. suratensis*) with cells at different stages of maturity.6000x

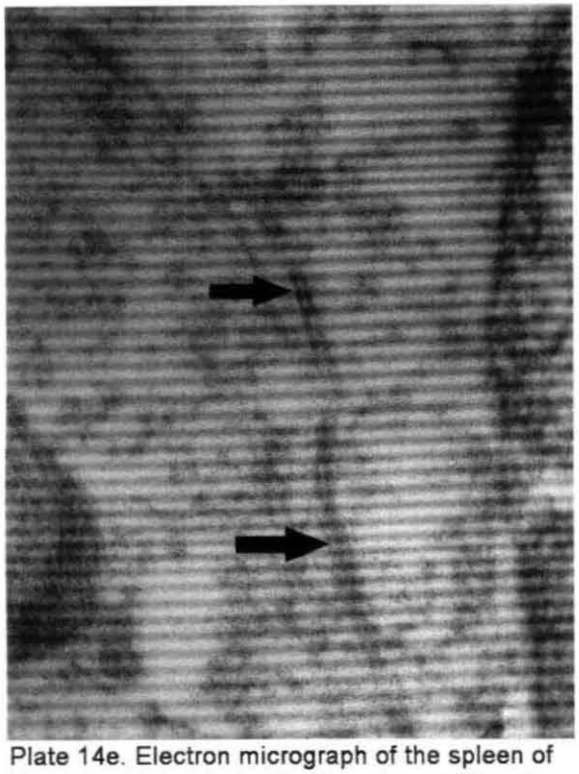


Plate 14e. Electron micrograph of the spleen of *E.suratensis* treated with aflatoxin revealing the necrosis of cell indicated by loss of organelles. Also note the discontinuous cell membrane. 80,000x

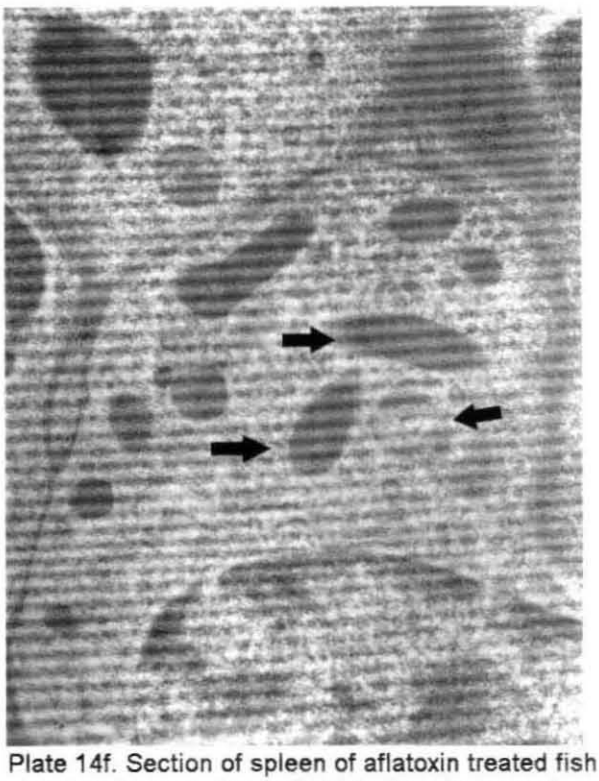


Plate 14f. Section of spleen of aflatoxin treated fish showing degenerating cell. Note the absence of cristae and granules in the mitochondria and swollen ribosomes.20,000x

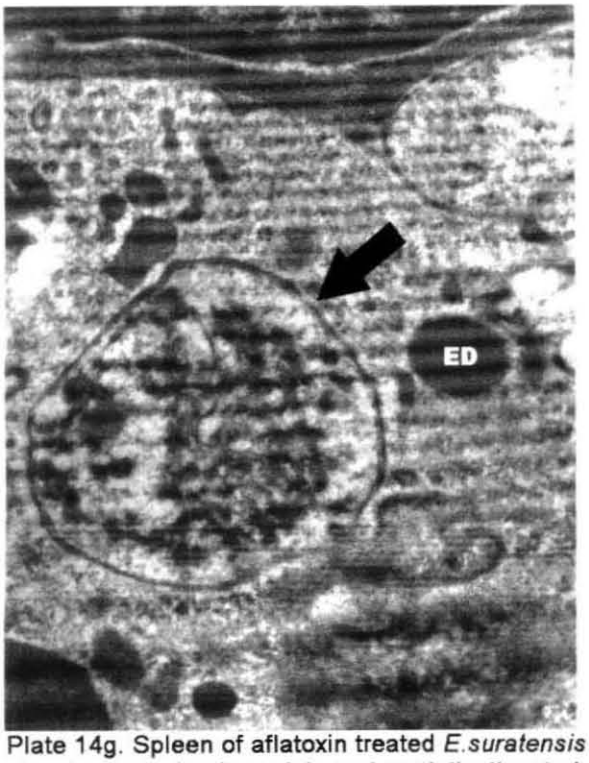


Plate 14g. Spleen of aflatoxin treated *E.suratensis* showing autophagic vesicle and partially digested organelles(arrow). Note the electron dense materials(ED)in the cytoplasm. 30,000x

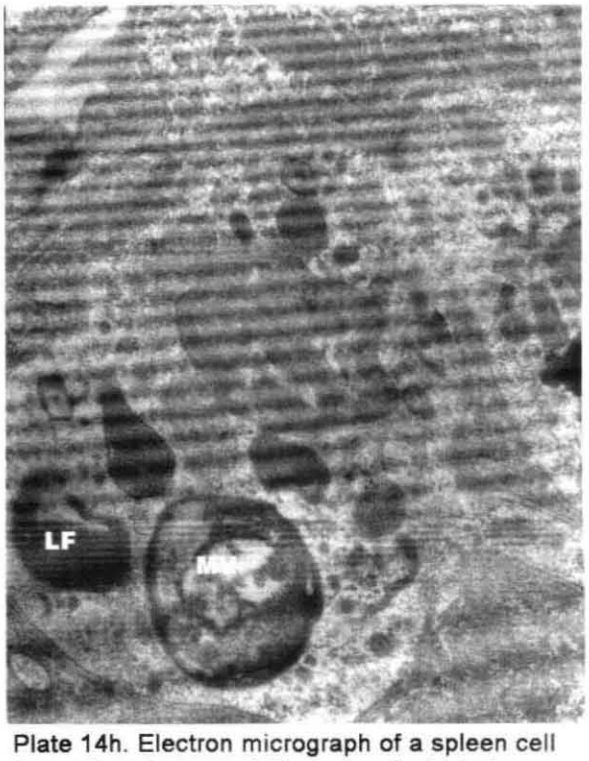


Plate 14h. Electron micrograph of a spleen cell from aflatoxin treated *E.suratensis* depicting multivesicular body(MV) and lipofuchsin(LF) formation.25,000x

Plate 15a. Section of thymus from control fish (*E. suratensis*).Note the presence of lymphoblasts in association with a blood sinus.4000x

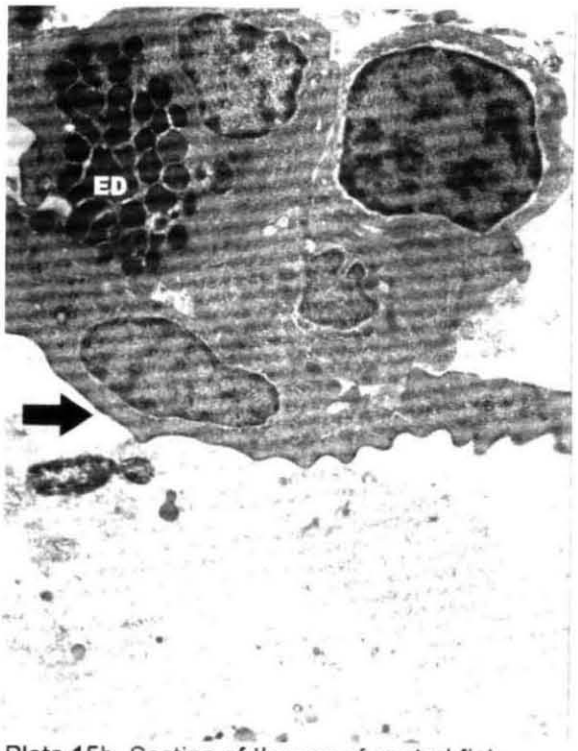


Plate 15b. Section of thymus of control fish (*E. suratensis*) showing an epithelial cell in close proximity with developing lymphoblasts. A cell containing abundant electron dense vesicles is also seen. 6000x

Plate 15c. Electron micrograph of the thymus of control fish(E. suratensis) showing lymphoblasts at different stages of maturity and also cells containing electron-dense vesicles. 6000x

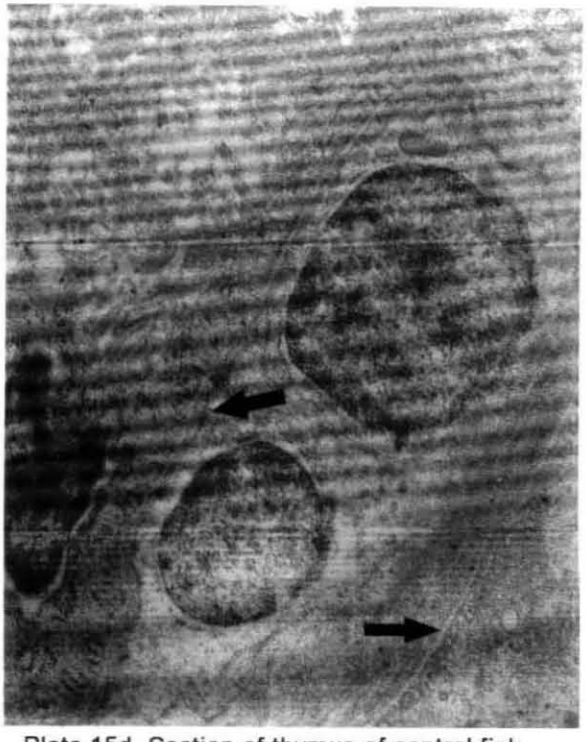


Plate 15d. Section of thymus of control fish (E. suratensis) with developing lymphoblasts in close proximity with epithelial like cell which have got numerous interdigitating processes. 15000x

4.2. STUDIES ON CADMIUM TOXICITY

4.2.1. Determination of LC_{50}

The 96 hr LC_{50} of cadmium in *Etroplus suratensis* was found to be 94 ppm.

4.2.2. Chronic Toxicity

One tenth of the LC_{50} was selected as the sublethal dose for conducting chronic toxicity study.

4.2.2.1. Behavioural Changes

The fishes exposed to sub lethal concentration of cadmium did not exhibit marked variations in the behavioural responses compared to the control group through out the experimental period.

4.2.2.2. Haematology

Total erythrocyte count, total leucocyte count, erythrocyte sedimentation rate (ESR), and packed cell volume (PCV) were determined from the treatment and the control group on all the sampling days viz, 14^{th}, 28^{th}, 42^{nd} and 56^{th} day of the rearing period and the results are presented in table 5.

4.2.2.2.1. Total Erythrocyte count

The total erythrocyte count was significantly ($p<0.01$) lower in the treatment group when compared to that in control during the first 28 days of cadmium exposure. However on 42^{nd} day it was significantly ($p<0.01$) higher in the treatment group when compared to that in control and on 56^{th} day there was no significant difference between control and treatment groups.

4.2.2.2.2. Total Leucocyte count

The total leucocyte count was significantly ($p<0.01$) lower in cadmium-exposed fishes than that in the control group on days 28 and 56 whereas on the 42^{nd} day, it was significantly ($p<0.01$) higher when compared to that in control. However, the general trend was a decrease in leucocyte count due to cadmium exposure.

Table 5. Haematological parameters of *E. suratensis* subjected to sublethal dose exposure to cadmium.

Total erythrocyte count ($\times 10^6$/ml)		
Days	Treatment	Control
14 **	4.7±0.133	6.6±0.062
28 **	5.33±0.042	9.22±0.336
42 **	8.19±0.096	4.77±0.085
56	5.6±0.115	5.36±0.130

Total leucocyte count ($\times 10^3$/ml)		
Days	Treatment	Control
14	33.83±2.08	40.33±1.53
28 **	17.83±1.04	47.83±1.61
42 **	65.33±1.26	31.67±1.15
56 **	18±1.32	24.83±0.76

ESR (mm/2hr)		
Days	Treatment	Control
14 *	9.67±1.528	6±1
28	10±1	8.67±0.577
42	10.67±0.577	11±1
56 **	19.67±1.528	9.33±1.155

PCV-Cadmium (%)		
Days	Treatment	Control
14 *	23.67±0.577	25.67±0.577
28	26±1	27±1
42 *	25.33±0.577	23.33±0.577
56 *	22.67±1.155	25.67±1.155

The mean values from treatment and control groups on each sampling day were compared by students' t-test.

* $p < .05$

** $p < .01$

4.2.2.2.3. Erythrocyte Sedimentation Rate (ESR)

The cadmium-exposed fishes exhibited a significantly higher ESR than the control fishes on the 14th ($p<0.05$) and 56th ($p<0.01$) day of the experiment. On the other two sampling days (days 28 and 42), there was no significant difference between control and treatment group although it was higher in the treatment group on day 28.

4.2.2.2.4. Packed Cell Volume (PCV)

A general decline in PCV was observed in cadmium-exposed fishes. In the treatment group, it was significantly ($p<0.05$) lower on the 14th and 56th day of the experiment whereas on the 42nd day it was significantly ($p<0.05$) higher than the control group.

4.2.2.3.Serum Factotrs

The serum factors studied include total proteins, albumin, globulin, albumin:globulin ratio and the enzymes, alkaline phosphatase, aspartate amino transaminase (AST) and alanine amino transaminase (ALT). The mean values of serum proteins and enzymes from control and treatment groups on the four sampling days are presented in tables 6 and 7 respectively.

4.2.2.2.1. Total Protein

The trend in total protein in treatment and control groups is depicted in figure 3. The total serum proteins in the control and treatment groups varied significantly only on the 24th day of the experiment wherein the treatment group registered a lower value when compared to the control. However, the cadmium exposure resulted in a general decline in serum proteins when compared to control.

4.2.2.2.2. Total Albumin

The total albumin values did not exhibit statistically significant difference between the control and the treatment groups throughout the experiment. However, the cadmium-exposed fishes registered a lower value than the control.

Table 6. Changes in serum proteins of *E. suratensis* exposed to sublethal dose of cadmium.

Total protein (g%)		
Days	Treatment	Control
14	1.69±0.064	1.78±0.083
28*	1.52±0.083	1.74±0.101
42	1.7±0.111	1.81±0.083
56	1.29±0.111	1.34±0.093

Albumin (g%)		
Days	Treatment	control
14	0.214±0.056	0.241±0.059
28	0.167±0.067	0.182±0.074
42	0.226±0.059	0.276±0.067
56	0.084±0.031	0.111±0.041

Globulin (g%)		
Days	Treatment	Control
14*	1.473±0.014	1.538±0.026
28*	1.355±0.076	1.56±0.029
42	1.473±0.157	1.534±0.150
56	1.211±0.083	1.227±0.052

A/G ratio		
Days	Treatment	Control
14	0.145±0.037	0.157±0.036
28	0.124±0.05	0.116±0.045
42	0.158±0.06	0.184±0.063
56	0.068±0.02	0.089±0.03

The mean values from treatment and control groups on each sampling day were compared by students' t-test.

* $p < .05$

** $p < .01$

Table 7. Changes in serum enzymes of *E. suratensis* exposed to sublethal dose of cadmium

AST /SGOT (enzyme units /ml)		
Days	Treatment	Control
14 **	32.7±2.38	11.19±3.151
28 **	4.2±3.429	24.58±2.38
42	7.62±3.636	12.04±2.194
56	3.78±2.577	2.78±1.71

ALT/ SGPT (enzyme units /ml)		
Days	Treatment	Control
14 **	32.82±1.466	10.06±2.726
28 **	3.39±1.625	12.82±1.746
42	7.87±2.292	11.39±2.006
56	3.3±1.288	1.77±1.591

Alkaline phosphatase (KA units/ml)		
Days	Treatment	Control
14 **	5.56±0.507	9.82±0.486
28 **	5.47±0.506	12.61±0.435
42 **	2.18±0.48	10.78±0.415
56	9.25±0.506	8.74±0.396

The mean values from treatment and control groups on each sampling day were compared by students' t-test.

* $p < .05$

** $p < .01$

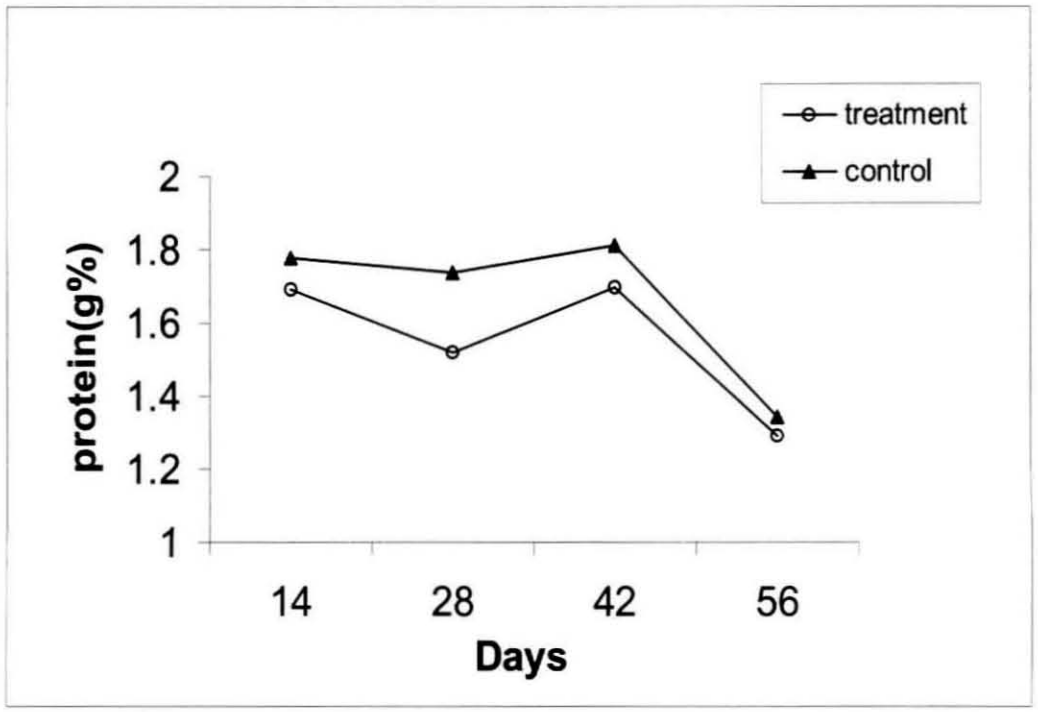


Figure 3. Changes in serum proteins of *E. suratensis* during exposure to cadmium.

4.2.2.2.3. Total Globulin

The total globulin value was significantly ($p<0.05$) lower in the treatment group than the control on 14th and 28th day of the experiment. On subsequent days also the cadmium-exposed fishes elicited a lower globulin value than control group although it was not statistically significant.

4.2.2.2.4. Albumin: Globulin Ratio (A/G ratio)

There was no significant difference in A/G ratio between the control and the treatment groups throughout the experimental period.

4.2.2.2.5. Alkaline phosphatase

The serum alkaline phosphatase activity in the cadmium-exposed fishes was significantly ($p<0.01$) lower than the control fishes on 14th, 28th and 42nd day of the experiment. On 56th day, the enzyme activity didn't register a significant difference between control and treatment group.

4.2.2.2.6. Aspartate aminotransaminase (AST) / SGOT

The serum AST activity was significantly ($p<0.01$) higher in cadmium treated fishes when compared to the control on 14th day whereas on the 28th day, it was significantly ($p<0.01$) lower in the treatment group. In the later half of the exposure period, the enzyme activity didn't elicit marked variation between the control and the treatment group.

4.2.2.2.7. Alanine aminotransaminase(ALT) / SGPT

The serum AST activity was significantly ($p<0.01$) higher in the treatment group compared to the control on the 14th day whereas it was significantly ($p<0.01$) lower in the treatment group than control on 28th day of the experiment.

During the later stages of the exposure period, there was no significant variation in the enzyme activity between control and treatment groups.

4.2.2.3. Histopathological Studies

The organs examined for histological alterations due to cadmium exposure include liver, kidney, spleen, thymus and gills.

4.2.2.3.1. Liver

The liver from the control fishes revealed structural details very similar to those already described under section 4.1.5.1.

In cadmium treated animals, in the initial stages of the experiment, the hepatocytes exhibited extensive vacuolation, the vacuoles pushed the nucleus to the periphery of the cells indicating fatty change in the hepatocytes. Occasionally hepatocytes showed necrotic changes. On subsequent days the liver sections revealed coagulative necrosis and proliferation of fibrous tissue. In advanced cases coagulative necrosis, fibrosis of parenchyma and moderate biliary proliferation were observed. (Plates 16a – 16b)

4.2.2.3.2. Kidney

In the control group, the kidney revealed structural details similar to those described under section 4.1.5.2. The convoluted tubule lined with columnar epithelium, glomeruli and Bowman's capsule were clearly seen without much structural alteration.

In cadmium exposed fishes desquamation of the epithelial cells of the tubules was evident in many areas. In some sections the tubular epithelial cells showed extensive vacoulation and necrosis. Remarkable changes were noticed in the glomeruli. In some cases there was accumulation of leukocytes around Bowman's capsule. The parietal layer of Bowman's capsule was thickened and glomerular capillaries appeared shrunken. There was also intercapillary thickening of the glomeruli. Some glomeruli elicited increased nuclearity indicating proliferation of

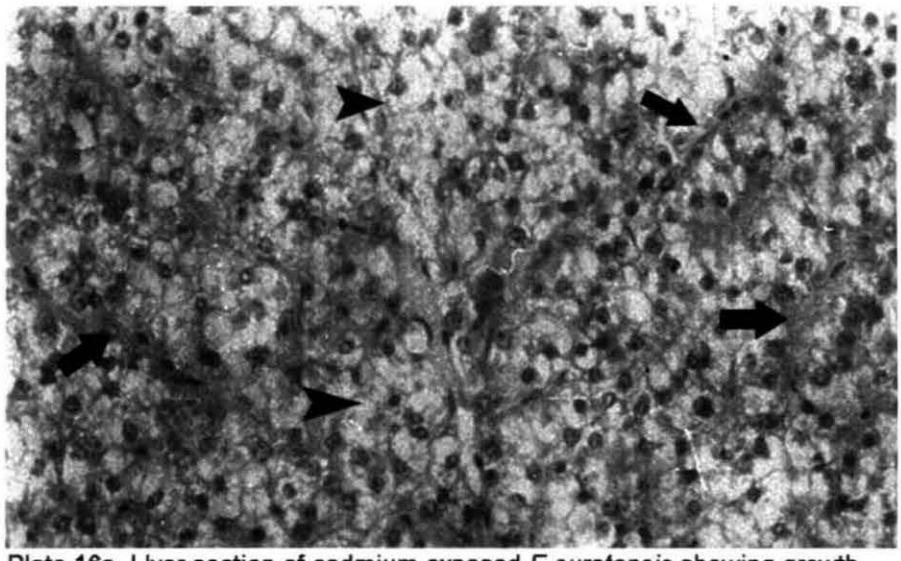


Plate 16a. LIver section of cadmium exposed *E suratensis* showing growth of fibrous tissue(arrow)in the parenchyma. Note the degenerative changes in the hepatocytes(arrow head). H&E 400x

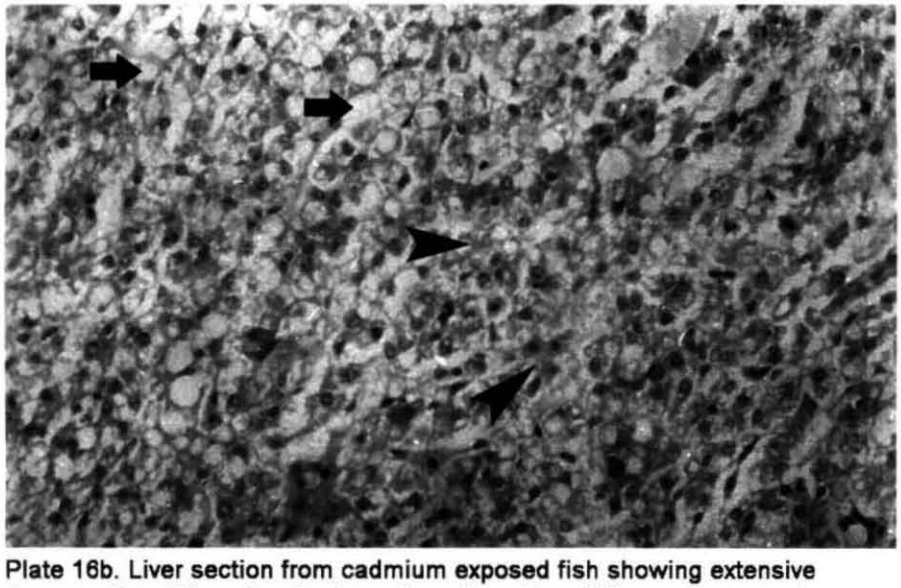


Plate 16b. Liver section from cadmium exposed fish showing extensive degeneration of hepatocytes(arrow), proliferation of fibrous tissue and biliary epithelium(arrow head). H&E 400x

mesenchymal cells. Glomeruli appeared shrunken in many areas. There was depletion of cells in the haemopoietic tissue. (Plates 17a – 17c)

4.2.2.3.3. Spleen

The spleen of the control group contained abundant ellipsoids with large number of cells similar to those already described under section 4.1.5.3. Lymphoid cells and erythropoietic tissue was also seen.

In cadmium treated fishes, there was depletion of cells in the ellipsoids compared to control. The lymphoid tissue also appeared depleted and the lymphocytes had shrunken, pyknotic nuclei indicating degenerative changes. (Plates 18a &18b)

4.2.2.3.4. Thymus

The thymus of the cadmium-exposed fishes showed severe degeneration and necrosis.

4.2.2.3.5. Gills

In the control group the structure of gill was intact. The primary and secondary lamellae had all the structure very similar to a normal structure. (Plate 19a)

In cadmium-exposed fishes, the secondary lamellae appeared elongated and some secondary lamellae were clubbed together and fused. Hyperplastic changes were seen at the base of the lamellae. Desquamation of the epithelial cells was also seen. (Plate Plates 19b &19c)

4.2.2.4. Ultrastructural Studies

Vital organs, liver, kidney, spleen and thymus were studied at the ultrastructural level to assess the impact of cadmium exposure.

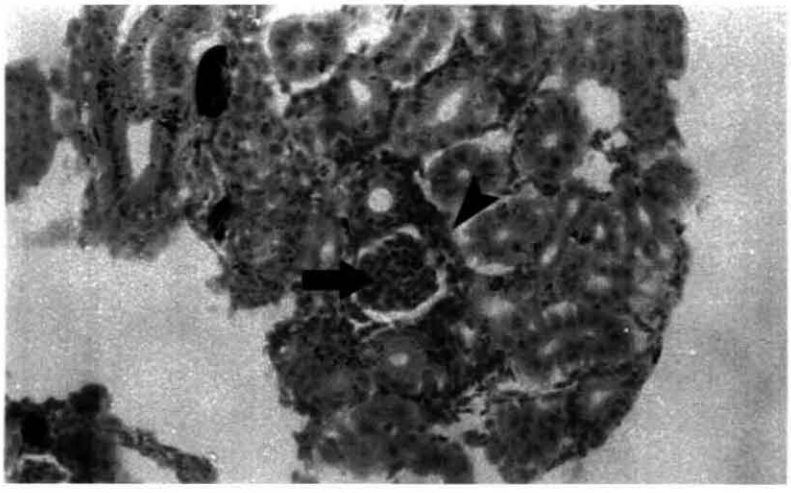


Plate 17a. Kidney of cadmium treated *E suratensis* showing increased nuclearity in the glomeruli(arrow). Note the thickening of bowman's capsule and infiltration of leucocytes(arrow head).H&E 400x

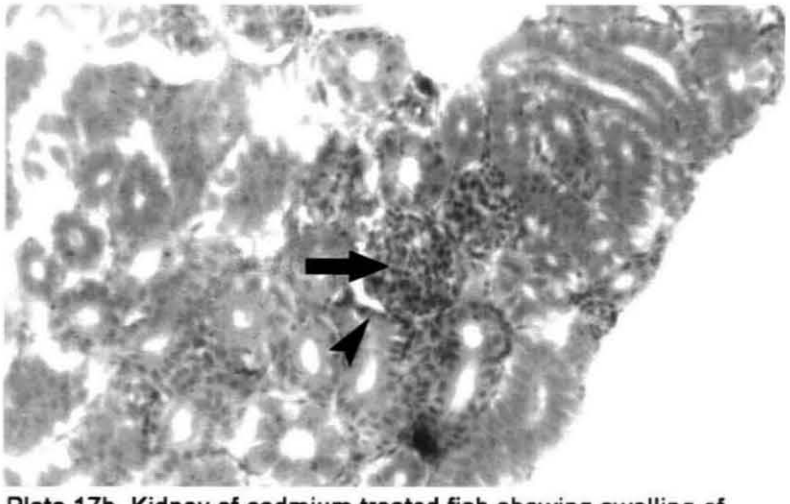


Plate 17b. Kidney of cadmium treated fish showing swelling of glomeruli with increased nuclearity(arrow). Periglomerular thickening is also seen(arrow head). H&E 400x

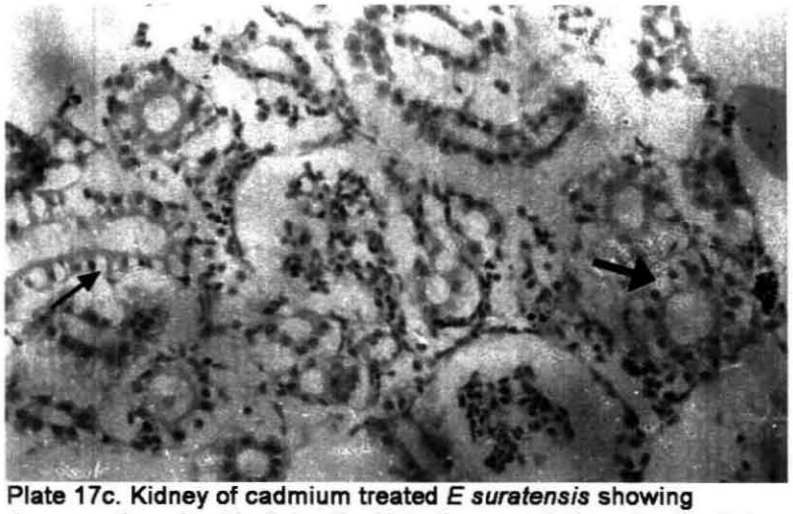


Plate 17c. Kidney of cadmium treated *E suratensis* showing degeneration of epithelial cells. Note the vacoulation of epithelial cells.H&E 400x

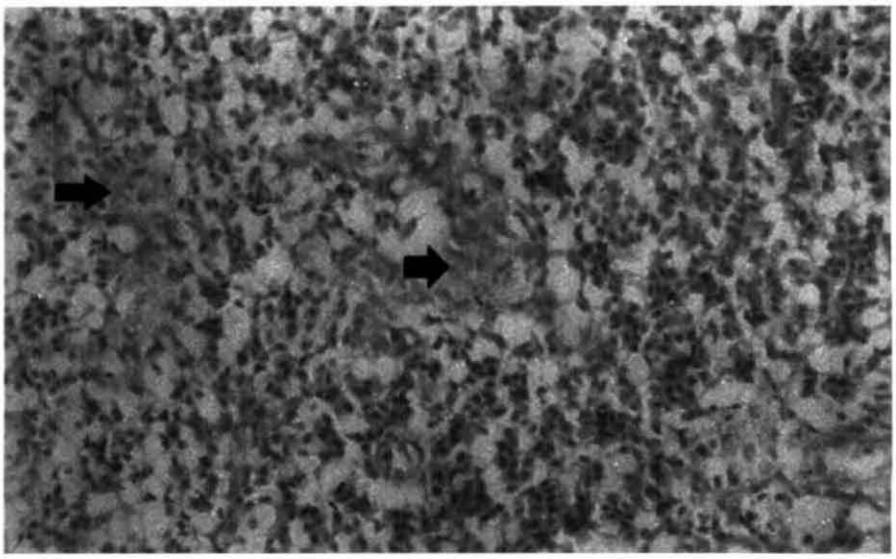


Plate 18a. Spleen of cadmium exposed *E. suratensis* . Note the depeltion of cells from the parenchyma. H&E 400x

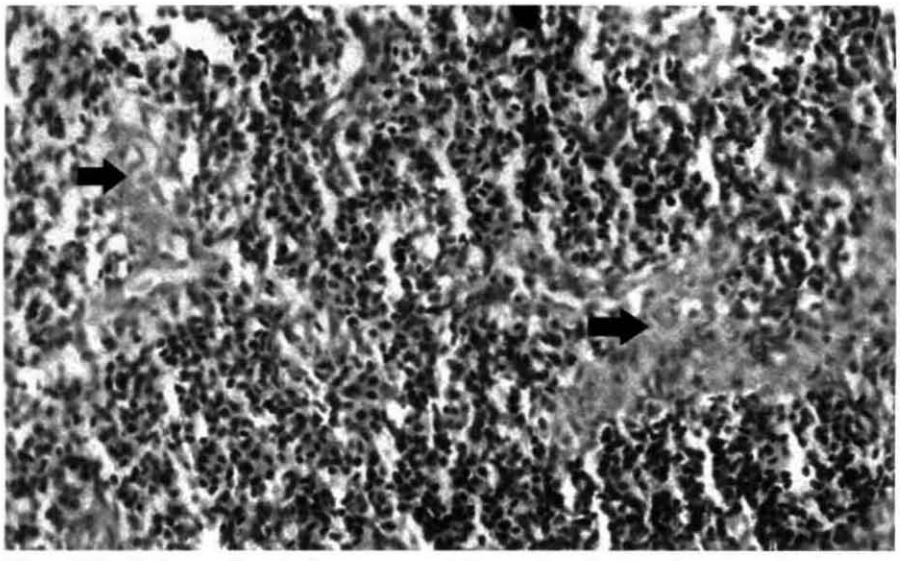


Plate 18b. Spleen of cadmium exposed *E.suratensis* showing depeltion of cells and degeneration of haemopoietic cells. H&E 400x

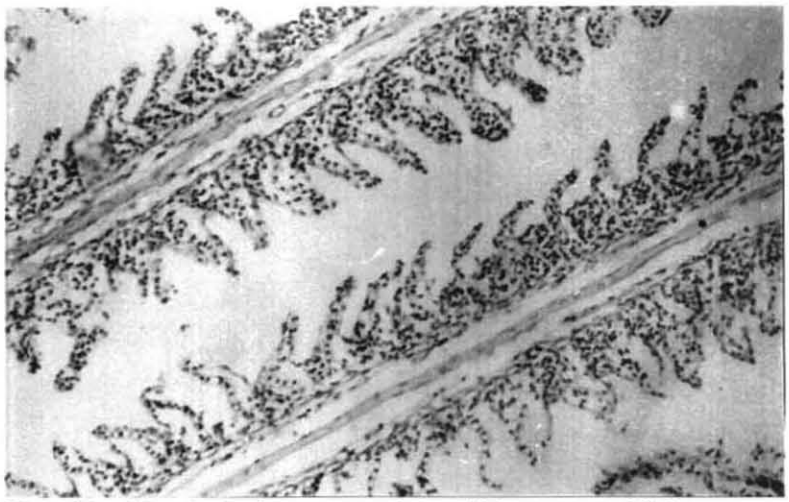


Plate 19a. Histological structure of the gill from control fish (*E.suratensis*)

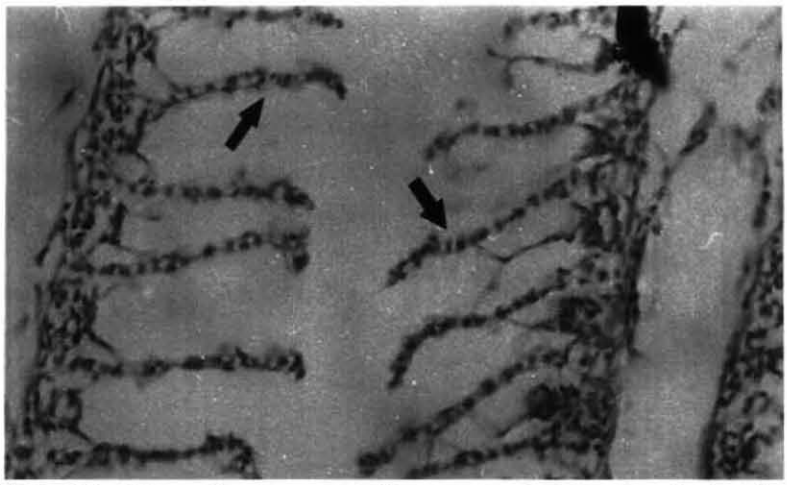


Plate 19b. Gill section from cadmium exposed *E. suratensis* showing the elongation of secondary lamellae. H&E

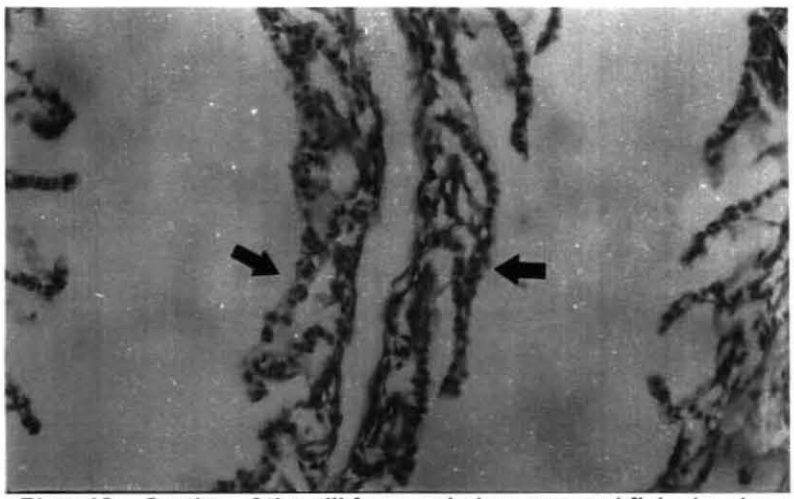


Plate 19c. Section of the gill from cadmium exposed fish showing the clubbing and fusion of secondary lamellae. H&E

4.2.2.4.1. Liver

The liver from the control fishes were normal with well developed rough and smooth endoplasmic reticulum, mitochondriae, golgi apparatus and nucleus.

The hepatocytes of cadmium treated fishes revealed remarkable changes from that of the control fishes. The endoplasmic reticulum (ER) of liver underwent conformational changes. Degranulation of RER was present in almost all samples examined. Progressive fragmentation of ER with the appearance of polysomes in the cytoplasm and dilatation of ER leading to vesicle formation/ vacoulation were evident. Many damaged mitochondria and other organelles were taken into autophagosomes by the encircling of ER. Clumping of nuclear chromatin was noticed in the nuclei. The ribosomes also appeared swollen. In advanced cases the nuclear membrane showed disruption and the ER showed severe fragmentation. Mitochondria lost its cristae, became condensed and shrunken. The desmosomes were detached and the hepatocytes lost its contact with neighbouring cells. (Plates 20a – 20d)

4.2.2.4.2. Kidney

In the control group the kidney cells had the normal structural details as already described under section 4.1.6.2.

In cadmium treated fishes, initially the microvilli of epithelial cells showed fusion. In later stages loss of microvilli was evident. The mitochondria were condensed. Swelling of mitochondrial membrane was noticed in many areas and mitochondria appeared swollen. The matrix was expanded and showed expansion of the inner compartment. There was thickening in the foot processes of the podocytes adhering to the endothelial surface. The haemopoietic tissue showed severe degeneration with nuclei of the cells appearing empty. Some nuclei appeared condensed and there was loss of architecture. In many areas the cells lost their nuclei, mitochondria became condensed and there was vacoulation indicating necrosis. The visceral epithelial cells showed nuclear invaginations and vacoulation. (Plates 21a – 21d)

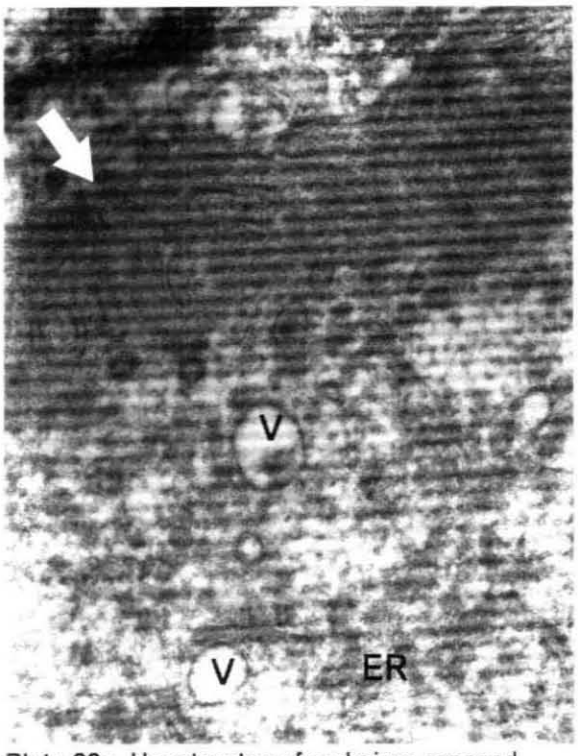


Plate 20a. Hepatocytes of cadmium exposed *E.suratensis* depicting the adhesion of microvillar processes, vacuolation(V), and the presence of swollen fragmented ER.There is also loss of ribosomes from ER.40,000x

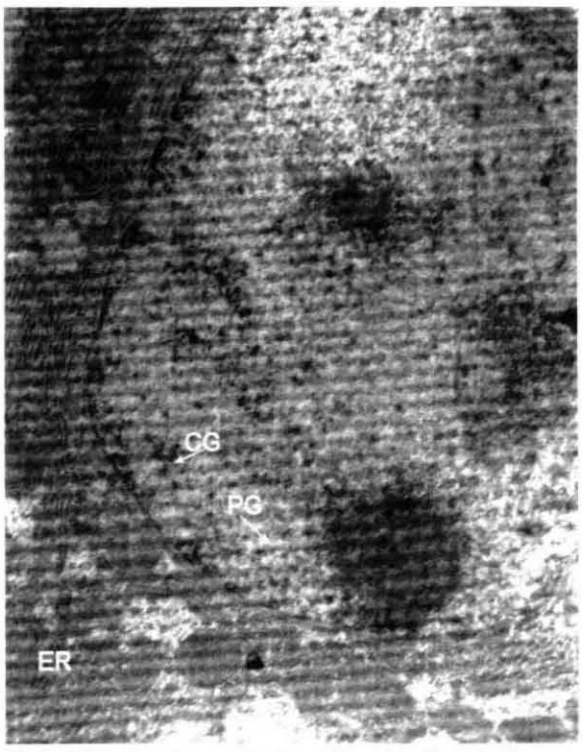
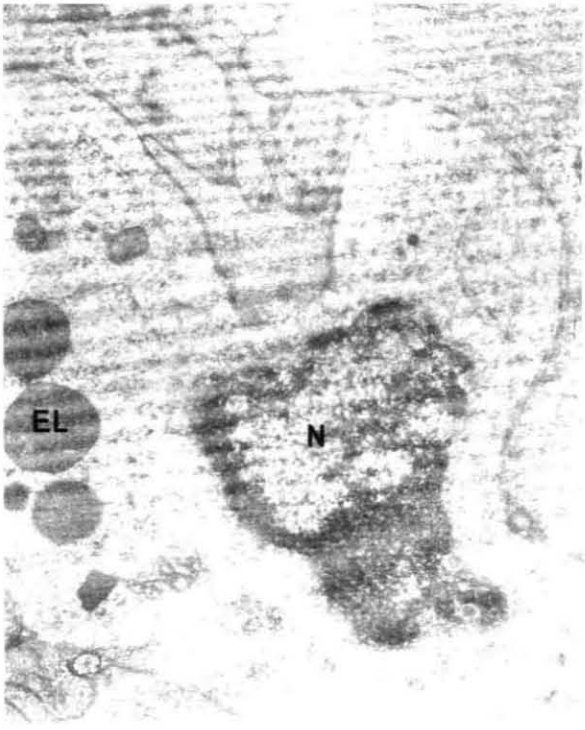


Plate 20b. Nucleus of a hepatocyte of cadmium exposed *E.suratensis* showing appearance of chromatin and perichromatin granules. Note the condensation of chromatin at ceratin points, fragmentation and degranulation of RER and loss of crsitae from mitochondria.15,000x

Plate 20c. Hepatocytes of cadmium exposed fish showing the detachment(arrow) of cells from each other.Also note the condensed mitochondria(M), fragmented ER and free swollen ribosomes(RB) in the cytoplasm.50,000x



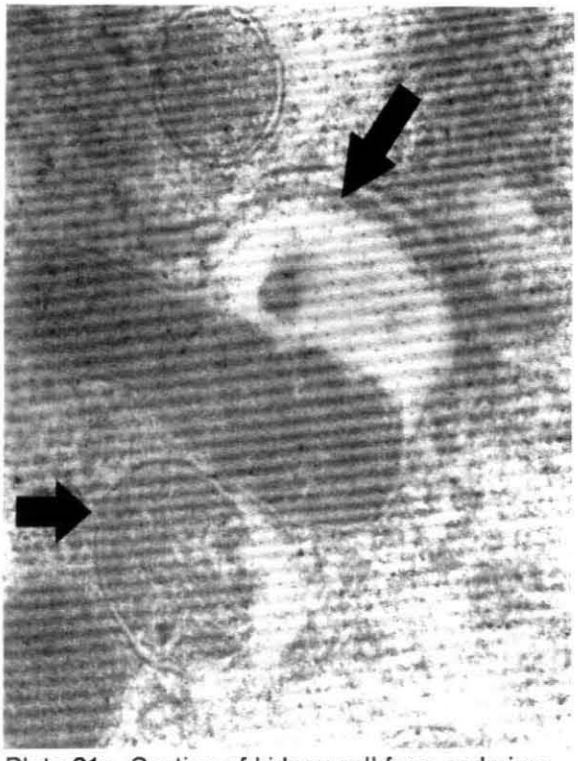


Plate 21a. Section of kidney cell from cadmium exposed *E.suratensis* showing autophagy of mitochondria. 60,000x

Plate 21b. Renal haemopoietic tissue of cadmium exposed *E.suratensis* undergoing degenerative and necrotic changes. Note the shrinkage and condensation of nucleus and absence of organelles in the cytoplasm. 5000x

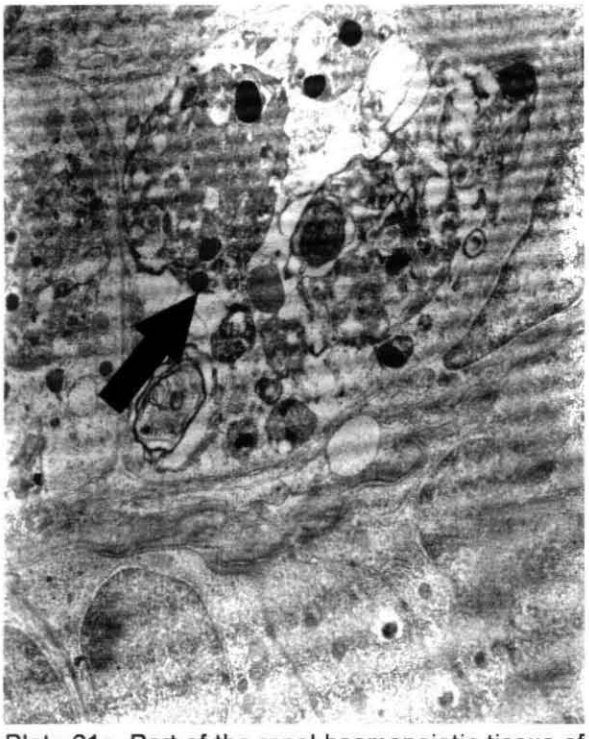


Plate 21c. Part of the renal haemopoietic tissue of *E. suratensis* exposed to cadmium revealing necrosed cells in the blood sinuses. 8000x

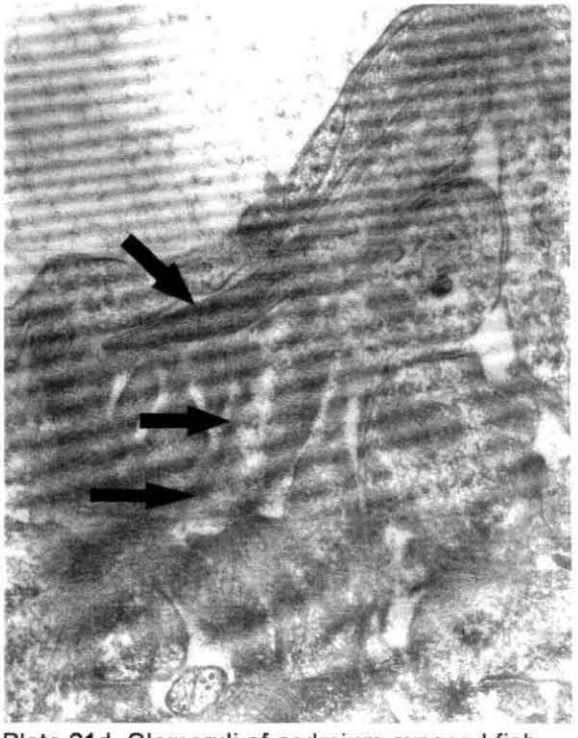


Plate 21d. Glomeruli of cadmium exposed fish. Note the presence of electron dense material in between the foot processes and basement membrane. 25,000x

4.2.2.4.3. Spleen

The spleen of control fishes contained developing lymphocytes, granulocytes and erythrocytes.

In the cadmium-exposed group, the lymphocytes showed degeneration. Many cells showed condensation of chromatin in the nucleus and shrinkage of the nucleus. The mitochondria were condensed and lost cristae. Cells were vacuolated and electron dense deposits were noticed in the vacuoles. (Plates 22a & 22b)

4.2.2.4.4. Thymus

In control fishes, the thymus indicated developing lymphoblasts in close proximity with epithelial type of cells. These cells had large tubular interdigitative cytoplasmic processes in close contact with developing lymphoblasts. Some of the cells contained membrane bound electron dense granules.

In cadmium-exposed fishes, the thymus contained vacant areas devoid of cells. The cells present had condensed mitochondria, clumped chromatin in the nucleus and the cells appeared detached. The cellular architecture of the thymus was lost. (Plates 23a & 23b)

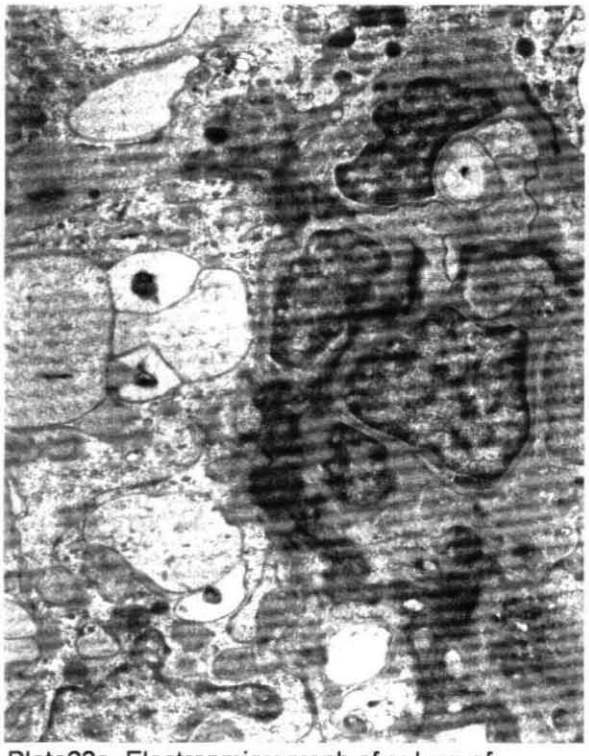


Plate22a. Electronmicrograph of spleen of cadmium exposed E. suratensis showing degenerating haemopoietic cells. 6000x

Plate 22b. Electronmicrograph of degenerating cells in the spleen of cadmium exposed *E. suratensis*. 8000x

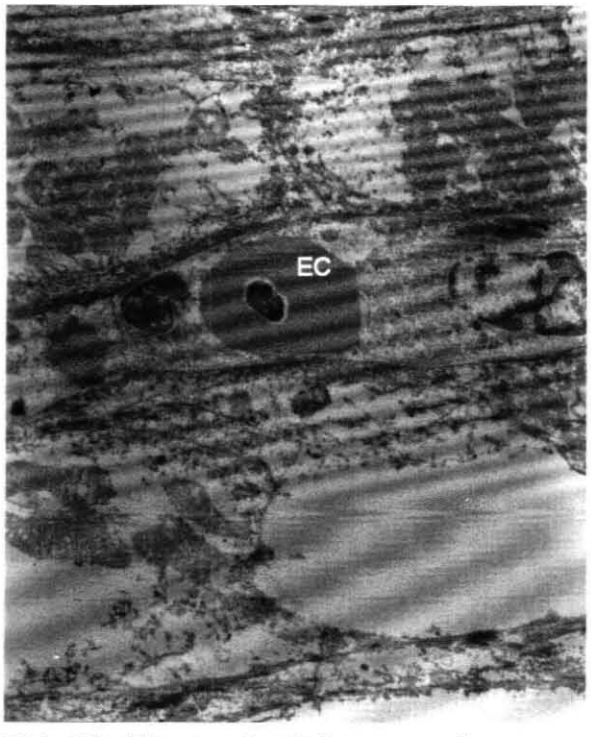


Plate 23a. Thymus of cadmium exposed *E. suratensis* showing loss of lymphoblasts and epithelial cells. Note the presence of intact erythrocyte(EC) in the sinus lumen. 5000x

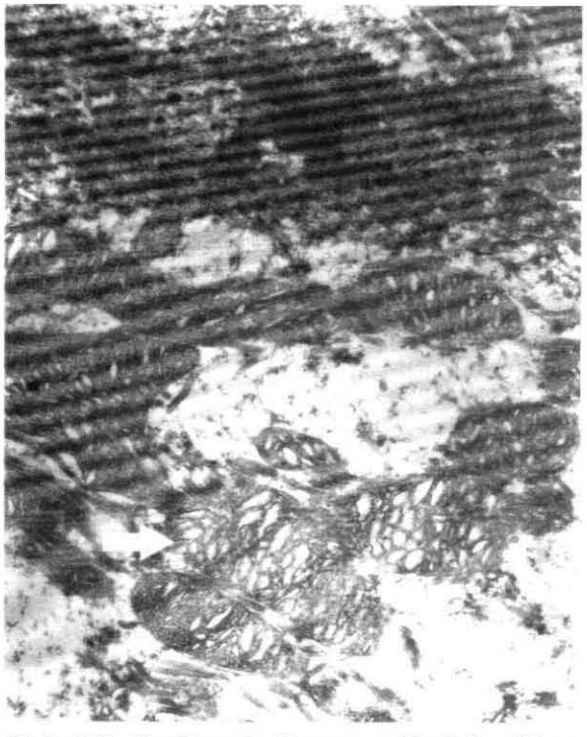


Plate 23b. Section of a thymus epithelial cell from cadmium exposed E. suratensis depicting the mitochondrial condensation. 15,000x

5. DISCUSSION

5.1. AFLATOXICOSIS

Though *E. suratensis* is being extensively used in polyculture systems in state like Kerala, where artificial feeding is also practiced, there is no systematic study on the effect of mycotoxins on this species. The present work is the first attempt to elucidate clinical and pathological changes due to mycotoxins. In aflatoxin fed fishes there was no apparent change in behaviour. The animals were feeding normally and there was no apparent mortality. This finding was contradictory to the earlier observation in many animals including fishes. In terrestrial animals, the presence of aflatoxin in the feed reduces the feed intake and causes higher mortality (Yoshida and Kamota, 1952; Asplin and Carnaghan, 1961; Chattopadhyay *et al.*, 1985; Chen *et al.*, 1985; Dalvi, 1986; Colvin *et al.*, 1989; Harvey *et al.*, 1989; Mukti and Kwatra, 1989; Roy *et al.*, 1989; Neldon Ortiz and Quereshi, 1992). Since no clinical symptoms or mortalitiy is recoreded in this species the farmer may be unaware of the serious problem of mycotoxin in their feeds and hence no precautionary measures are taken to avoid these toxins. Thus, in the long run, due to the prolonged and unabated toxic insults of aflatoxin, the immunocompromised fishes may become succeptible to other diseases and consequently crop losses can occur.

5.1.1. Haematology

The haematological studies in aflatoxin treated fishes revealed remarkable changes in erythrocyte count, leucocyte count, ESR and PCV. There is only scanty information about the effect of aflatoxin on haematological parameters of fishes. According to Svobodova and Piskae (1980) and Svobodova *et al.* (1982) the haematological parameters are not affected by aflatoxin. However, in channel catfish an acute dose of 12mg AFB_1/kg body weight caused a marked reduction in erythrocyte count (Janrarotai *et al.*, 1990). In a ten-week feeding experiment with subacute levels of AFB_1 (100 – 10,000ppb) Janrarotai and Lovell (1990) recorded a significant reduction in erythrocyte count at the highest dose tried. A similar result was also obtained by Plumb *et al.* (1986). In the present study also there was a

decrease in the erythrocyte count in the first two weeks but subsequently there was increase in the number of erythrocytes. The studies in other vertebrates also indicate anaemia and reduction in haematocrit values as a result of aflatoxin treatment (Wannop, 1961; Brown and Abrams,1965; Tung *et al.*, 1975). The present observation is different from earlier studies (Loc.cit). This is the first observation in *E. suratensis* on aflatoxicosis. Though there was reduction in the erythrocyte count, the increase on subsequent days indicates a compensatory response from the animal against the adverse effect of aflatoxin. A detailed study is required for elucidating the cause and mechanism for the increase seen in later days of experiment.

The leucocyte count of aflatoxin treated fishes did not vary till the second week of the experiment. After the second week it was significantly higher than the control. Though there are reports on leucocyte decrease in fishes when they are fed aflatoxin containing feed (Janrarotai *et al.*, 1990; George, 1998), studies have shown that sub lethal doses of aflatoxin cause increase in the total leukocyte count in channel catfish, Indian catfish and in shrimps (Parashari and Saxena, 1983; Janrarotai and Lovell, 1990; Boonyaratpalin *et al.*, 2001). In animals like goat and chicken aflatoxin has caused an increase in the leukocyte counts (Wannop, 1961; Anilkumar and Rajan, 1987). The results of the current study also agree with the observation that aflatoxin causes an increase in leukocyte count. However, Sahoo and Mukherjee (2002) are of the opinion that, in rohu, aflatoxin does not cause any change in leukocyte count 60 days after injection (ip) of the toxin. As the exposure route in most of the other studies is feed, the extent of toxic impacts due to aflatoxin on leucocyte count may be influenced by the route of exposure.

The ESR showed significant difference between the control and the aflatoxin treated groups. The ESR indicates the viscosity of plasma as well as stickiness of erythrocyte, which depends on the plasma protein concentration. The serum protein though showed a decreasing trend in the treatment group, the ESR values did not reflect the change. Hence, the ESR values could not be correlated with the aflatoxin treatment. Though there was significant difference between control and treatment groups at different points of time the changes were not uniform.

The PCV was significantly higher in the treatment group at the later stages of the experiment. This was in conformity with the increase in erythrocytes seen in treatment group. In channel catfish, aflatoxin at acute and sub lethal doses cause lowering of haematocrit (Janrarotai and Lovell, 1990., Janrarotai *et al.*, 1990; Janrarotai, 1991). In tilapia also the same trend is noticed (Anh Tuan *et al.*, 2002). Even though, results of the present study are contrary to the observations loc.cited, it was in conformity with the variation in erythrocyte count. This may be attributed to the inherent fluctuations and variation in the normal range of erythrocyte count in fishes (Schaperclaus, 1986; Ellis *et al.*, 1989)

5.1.2. Serum Factors

Total serum protein values in the treatment group started showing significant decrease from the third fortnight onwards and was very low in the fourth fortnight. The albumin values though initially showed an increase in the aflatoxin treated group, it also fell significantly on subsequent days. The globulin values also showed a decrease in treatment group in the later stages of the experiment. The decrease in total protein values is due to the decrease in albumin and globulin. The A/G ratio also showed a significant reduction.

The aflatoxin treatment in terrestrial animals and birds causes hypoproteinemia (Brown and Abram, 1965; Baker and Green, 1987; Singh *et al.*, 1987; Huff *et al.*, 1988; Harvey *et al.*, 1988). Saber (1995) has reported a reduction in total protein, albumin and globulin in aflatoxin treated *Tilapia nilotica*. Sahoo and Mukherjee (1999) have also reported a reduction in total protein in rohu treated with aflatoxin. George (1998) has reported a reduction in total protein and albumin levels in rohu treated with aflatoxin. In the present study also there was reduction in total protein, albumin and globulin, which is in conformity with the earlier observations in other animals. Aflatoxin affects liver, which is the major locus of serum protein synthesis in animals and thus the reduction in serum protein can be attributed to the severe damage to the liver. The reduction in globulin values will have an effect on the resistance of the fish against infections since, factors responsible for specific and non-specific immune responses are present in globulins. Hence aflatoxin will have far reaching effect on the health of the fish.

Three enzyme profiles in the serum were studied in the aflatoxin treated animals. They were alkaline phosphatase, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT).

The alkaline phosphatase was significantly higher in aflatoxin treated fishes. The liver biliary system and liver endothelial cells are rich sources of alkaline phosphatase in fish (Teh and Hinton, 1993). The liver biliary hyperplasia was a major lesion in all aflatoxin treated fishes. Increase in serum alkaline phosphatase can be very well attributed to the increase in biliary tissue in the liver and this finding is highly significant because by estimating alkaline phosphatase in the serum, it may be possible to detect biliary hyperplasia.

AST and ALT, the two liver enzymes also showed significant increase in aflatoxin treated fishes. ALT is a cytoplasmic transaminase present in hepatic cells and is released from cytoplasm of injured liver cells in moderate injuries whereas AST is located in mitochondria and is released in more extensive injuries. These two enzymes are universally used to diagnose liver injury (Freedland and Kramer, 1970; Thomson, 1974; Farber and El-Mofty, 1975). The present study also revealed considerable hepatocellular damage including severe mitochondrial damage, which explains the increase in the levels of these enzymes. Blood levels of transaminases in fishes are subject to strong variations. The stress conditions caused by increase in environmental temperature, harmful substances and excessive protein metabolism cause elevation of transaminases in the blood of fishes (Duong, 1974; Spangenberg and Schreckenbach, 1984). These findings show that ALT and AST are good indicators of stress including mycotoxins. Though there is very little information on aflatoxin induced serum transaminase changes, the present results are highly important.

5.1.3. Immunomodulation

Assessment of cell mediated immunity (CMI) and humoral immunity was also carried out in the aflatoxin treated fishes. There was a reduction in CMI response as evidenced by the reduction in PHA response in treatment group. George (1998) has reported CMI suppression in aflatoxin treated rohu. In goat, aflatoxin feeding has produced poor PHA response (Anilkumar and Rajan, 1987). PHA response was found to be lowered in tumour bearing green turtles (Work *et al.*, 2001). The role of aflatoxin in causing immunosuppression in fishes has not been studied extensively. The present observation also indicates that in fishes CMI is affected by aflatoxin. The immune responses in fishes are highly influenced by external factors like nutrition, pollutants and toxins (Balzer and Wolke, 1984; Li and Lovell, 1985). Thus aflatoxin may be a major immuno-suppressing factor in fishes.

The humoral immune response as evinced by antibody titre against *A. hydrophila* indicated a lower titre in aflatoxin fed fishes. Though the depression was not enough to create a statistical significance, still the trend was in conformation with the previous results of Arkoosh and Kattari (1987), George (1998), Sahoo and Mukherjee, (2002).

5.1.4. Vital organs

5.1.4.1. Liver

The liver from control fish had a structure very similar to the normal liver structure described by Shore and Jones (1889) and Hampton *et al.* (1988). In this arrangement hepatocytes have their bases directed towards sinusoids and their tapered apices form the wall of the initial portion of the biliary system, the biliary canaliculi. The small biliary epithelial cells are in direct contact with hepatocytes and have junctions with them (Hinton *et al.*, 1984; Hampton *et al.*, 1988).

In the initial stages of aflatoxin exposure, the hepatocytes showed vacoulation and the stromal connective tissue became prominent. This vacoulation indicates the hepatocellular degeneration due to acute cellular swelling and accumulation of neutral fat. Liver is the primary locus of histological alteration in

toxicity (Meyers and Hendricks, 1985; Murchelano and Wolke, 1985; Wolke *et al.*, 1985; Myers *et al.*, 1987; Harshbarger and Clark, 1990; Kranz and Dethlefsen, 1990; Vogelbein *et al.*, 1990) because of its key role in the metabolism and detoxification of toxicants. It is also the major site of cytochrome P450 mediated mixed function oxidase system in teleosts (Stegeman *et al.*, 1979). Swelling of cells is an important and common response to cellular injuries of all types (Thomson, 1984). Focal coagulative necrosis and loss of architecture of the hepatic parenchyma were evident as the exposure time increased. Coagulative necrosis results from sudden cessation of blood flow to the liver by toxic agents. This is a feature in liver associated with toxicity and in a number of cases of toxicity in fishes, coagulative hepatocellular necrosis has been identified as a biomarker (Wyllie *et al*, 1980; Meyers and Hendricks, 1985; Pilot, 1988). Coagulative necrosis, fatty change and other degenerative changes have also been observed in association with aflatoxicosis in tilapia, coho salmon and channel catfish (Bruenger, 1982; Bruenger and Gruel, 1982; Teresa *et al.*, 1987; Jantrarotoi and Lovell, 1990; Jantrarotai *et al.*, 1990). Chavez *et al.* (1994) have reported that in *O.niloticus* aflatoxin produced nuclear hypertrophy, cellular atrophy, fatty liver and leucocytic infiltration. Thus it is clear that, fatty change and coagulative necrosis observed in *E. suratensis* is due to the effect of aflatoxin.

Fibroblastic proliferation and biliary proliferation were observed as the exposure time to aflatoxin increased. There was infiltration of mononuclear leucocytes around proliferating biliary ducts. Later the proliferation of the biliary epithelium increased and occupied a major portion of the liver parenchyma along with fibrosis and necrosis. The leucocytic infiltration seen in the present case was earlier reported by Chavez *et al.* (1994). Biliary epithelial cells are responsive to various types of insults. Proliferation of biliary passageways occurs as a chronic response to liver injury. When fish are exposed to carcinogens, alterations in the biliary system accompany changes in the hepatocytes. Proliferation of passageways is closely associated with extensive fibroblast proliferation and connective tissue increase (Hinton and Lauren, 1990). Aggregation of macrophages in proximity to biliary epithelial cells is a common factor in toxicity (Wolke *et al.*, 1985). Hyperplasia of the biliary epithelium is a common lesion in wild fish exposed to chemical contaminants (Murchelano and Wolke, 1985; Hayes *et al.*, 1990).

In animals exposed for a longer time, the liver surface showed black spots as well as raised white patches. These livers on histology revealed areas of hepatic cell regeneration, which were evidenced by the hepatocytes having large amount of basophilic cytoplasm and vesicular nuclei. There were basophilic foci of large plum cells in hepatic parenchyma. A large number of plum hepatocytes with different shapes were also noticed. Some of the cells appeared hypertrophied with large nuclei and some contained multiple nuclei. Hyperplasia is indicative of extensive prior necrosis from toxicants. The regenerative foci is a histopathological biomarker of a recent toxic insult (Kent *et al.*, 1988). The enlargement of hepatocytes is a hypertrophy or hepatocytomegaly characterized by organelle hyperplasia and enlarged cellular diameter without nuclear changes. Another type of hepatocytomegaly is characterized by marked cellular and nuclear enlargement and is termed megalocytosis or megalocyte hepatosis (Hinton, 1993). These type of lesions are found in fish living in polluted environments and it is a manifestation of chronic toxicity. Such lesions have been described by Hendricks *et al.*, 1981; Hinton *et al.*, 1988a&b; Kent *et al.*, 1988 and Myers *et al.*, 1990 in association with pollution. The basophilc foci and megalocyte foci are thought to represent formation of hepatic adenoma (Hendricks *et al.*, 1984; Nunez *et al.*, 1991). These lesions may be microscopic in size and the hepatocytes appear essentially normal retaining their normal architecture (Myers *et al.*, 1987; Nunez *et al.*, 1991). Baumann *et al.* (1990) is of the view that focal hepatocellular alterations are intermediate changes that bridge normal hepatocytes to hepatocellular carcinoma and they termed this as adenoma. The early stages in the development of neoplasia is characterized by the formation of foci of cellular alteration. These foci includes those with staining alterations and also cells with morphological alterations (Hinton, 1993). Many of the preneoplastic foci appear as basophilic, eosinophilc or clear cell foci (Egami *et al.*, 1981; Couch and Courtney, 1987; Hinton *et al.*, 1992). In the present work, large number of megalocytosis as well as basophilic foci were seen after six weeks of aflatoxin treatment and the megalocytes were having multiple nuclei. All these indicate either a neoplasmic transformation of the hepatocytes or a pleneoplastic lesion or adenoma as described by Baumann *et al.* (1990). In three fishes, which had black spots on the liver, the liver parenchyma was invaded by pleomorphic polyhedral basophilc cells having hyperchromatic nuclei and high mitotic index. These cell sheets invaded into the normal parenchyma where the hepatocytes underwent severe degeneration and

necrosis. The cells of this growth appeared to be very similar to the cells of the carcinoma in rainbow trout described by Majeed *et al* (1984) and hepatocellular carcinoma of trout (Nunez *et al.*, 1991). The invasiveness and rapid proliferation of hepatocellular carcinoma caused severe compression of surrounding hepatocytes and they have grown in large sheets or trabeculae and less frequently in a glandular pattern. Many cells appeared polyhedral in shape and there were numerous mitotic figures. The histological picture of the growth is very similar to hepatocellular carcinoma in fish as described by Hendricks *et al.* (1984), Myers *et al.* (1987), Couch and Courtney (1987), Hinton *et al.*, (1988a&b) and Vogelbein *et al.* (1990).

The ultrastructural changes in hepatocytes include vacoulation, mitochondrial damage, autophagia, appearance of liposomes, peoxisomes and multivesicular bodies; degranulation, dilatation and proliferation of ER; damage to desmosomes and loss of cell attachment. These ultrastructural changes are in conformity with the biochemical and histological changes observed. Scarpelli *et al* (1963) has reported proliferation and dilatation of ER in rainbow trouts exposed to aflatoxins. The hepatocytes are the site of detoxification of xenobiotics and the organelle responsible for detoxification is smooth endoplasmic reticulum (SER). The SER undergoes proliferation in response to increased demand for biotransformation of xenobiotics. (Zweifach *et al*,1965,. La Via and Hill,1971., Cheville,1983). Proliferation of ER causes megalocytosis (Hinton, 1993). Thus,the hepatocytomegaly observed in the histology slides can be attributed to the proliferation of ER seen in the electronmicrographs. Later severe changes were noticed in the architecture of the organelle. The nucleus was displaced to the periphery of the cells with the development of vesicles. Nunez *et al.* (1991) has reported pleomorphic nuclei, large nucleoli, dilated ER and increased numbers of lysosomes in rainbow trouts fed with aflatoxin. Similar changes are observed in the present study also like, dilatation of RER in hepatocytes, presence of perichromatin and chromatin granules in the nucleus and increase in the heterochromatin content of the nucleus. In organophosphate/ pesticide toxicity, the RER of hepatocytes in rainbow trout undergoes loss of structural integrity. When lower doses of pesticides are given, RER shows proliferation (Arnold *et al.*, 1995). The present study also revealed such changes. Fragmentation and degranulation of ER are considered to be the result of peroxidation of unsaturated fatty acids in the membranes by free radicals generated.

This also results in the separation of desmosomes. The autophagy, multivesicular bodies and the mitochondrial changes seen in the hepatocytes are also the result of cell injury (Zweifach *et al.*, 1965; La Via and Hill, 1971; Cheville, 1983; Thomson, 1984). The changes like nuclear protein inclusions, condensation of organelles and multivesicular bodies were more pronounced in hepatocytes of *E suratensis* compared to the earlier reports. This indicates that aflatoxin has got severe effect on pearl spot livers compared to carps.

The initial studies of aflatoxicosis in fish were mostly carried out in trout because in trout AFB_1 produced hepatocarcinoma (Ashley and Halver, 1961; Halver *et al.*, 1962; Halver, 1969; Ashley, 1970; Sinnhuber *et al.*, 1977; Wales, 1979; Hendricks *et al.*, 1979; Rasmussen *et al.*, 1986; Nunez *et al.*, 1990, 1991; Ngethe *et al.*, 1993; Sarcione and Black, 1994). Other than trouts, tilapia is considered to be susceptible (Haller and Roberts, 1980). Hence these two species were considered to be the animal model for the carcinogenic studies. *E. suratensis* is an asian cichlid which is a potential species for aquaculture. It is shown that this species is highly susceptible to mycotoxins and it is easy to induce carcinomas using aflatoxins. Among the other cultivated species studied, carps are known to be comparatively resistant to the carcinogenic effects of aflatoxins. Therefore there is much scope for using *Etroplus suratensis* for the study of hepatocarcinogenesis. The present study has established that these fishes respond to aflatoxins very quickly.

5.1.4.2. Kidney

In the present study the kidney showed severe histomorphological changes in response to aflatoxin treatment. Epithelial cells of the proximal convoluted tubules lost their brush border appearance. The cells became shrunken and desquamated. Some of them had vacuoles inside the cytoplasm and many of them desquamated into the lumen. Changes in the glomeruli were also observed. The glomerular capillaries showed focal thickening and glomeruli developed adhesions with the parietal layer of Bowman's capsule. The basement membrane of Bowman's capsule became thickened and the parietal epithelial layer proliferated. Fibrosis of Bowman's capsule was also noticed. The haemopoietic tissue also showed degenerative changes.

Kidneys in fishes have got critical functions as it receives large volume of blood flow from both the renal and portal venous system and the renal arteries. Most of the xenobiotics are excreted through glomerular filtration and tubular secretion. Hence it is assumed that the kidney is at high risk from toxiclogical attack (Pritchard and Renfro, 1982; Walker, 1987). The presence of xenobiotic metabolic enzymes like aryl hydrocarbon hydroxylase and cytochrome *P* 450 mono-oxygenase in kidneys of fishes indicate that kidney is exposed to xenobiotics (Bend *et al.*, 1973; Stegemen *et al.*, 1979; Lindstrom-Seppa *et al.*, 1981; Payne *et al.*, 1984; Williams *et al.*, 1986). Though histopathological studies in kidneys of fishes exposed to toxicants are scanty, Hinton (1993) is of the opinion that lesions like tubular necrosis, glomerular changes and eosinophilic droplets in proximal tubules are indicative of prior exposure to toxicants. Chromosomal aberrations in the kidney cells of cyprinids within 48 hrs of exposure to aflatoxin were observed by Al Sabti (1985). Verma and Pandey (1987) have reported the appearance of pleomorphic lymphocytes in the kidney tissue of *Channa punctatus* on exposure to aflatoxin. Aflatoxin affected the kidneys of channel catfish where the sinusoids of the kidney contained dilated haemopoietic areas with large number of immature erythrocytes (Jantrarotoi *et al.*, 1990; Jantrarotoi and Lovell, 1990; Jantrarotoi, 1991). In tilapia severe changes were observed in the kidney during aflatoxicosis (Chavez *et al.*, 1994). The glomeruli underwent shrinkage and there was melanosis in the kidney. George (1998) also reported glomerular and tubular lesions in kidneys of rohu exposed to aflatoxin. Sahoo *et al.* (2001) have reported severe necrotic changes in kidney of rohu. The glomerular changes are important because such changes are noticed in the glomeruli of fishes during various types of insults and the reasons for capillary thickening in fishes has not been elucidated so far (Roberts, 1989). Glomerular capillary thickening is a main feature of glomerular nephritis in higher vertebrates. This is frequently associated with either immunocomplex mediated or autoimmune mediated inflammatory reactions (Jones and Hunt, 1983). The present observations in pearl spot suggest the possibility of toxicants like aflatoxins in causing such lesions in fishes.

Aflatoxin produced severe degenerative changes in the epithelial cells of kidney. This observation is supported by the ultrastructural studies, which revealed changes in RER, mitochondria, nuclei and lysosomes of the cells. The ER fragmentation, loss of ribosomes and various mitochondrial changes seen in hepatocytes were also seen in epithelial cells. The nuclei showed heterochromatin formation and conformational changes in the nuclear membrane. All these changes indicate failure in protein synthesis, respiratory metabolism and consequently total loss of kidney function. These electron micrographs points to a shift in the ionic metabolism because kidneys are involved in the ionic balance of fish (Trump *et al.*, 1975). The changes in RER and autophagy observed in the present study had identical effects and causes as described in the hepatocytes. The aflatoxin also caused degenerative changes in the haemopoietic tissue. Electron micrographs revealed extensive changes in cell organelles.

These findings show that, kidney is also one of the target organs of aflatoxin toxicity. This is further substantiated by the renal tumours in tilapia during aflatoxicosis (Haller and Roberts, 1980). This study in pearl spot confirms that aflatoxin affects the renal tissues of fishes.

5.1.4.3. Spleen

In the spleen of aflatoxin treated fishes, the ellipsoids were depleted of macrophages. The spleenic white pulp also contained fewer lymphocytes and the lymphocytes present were shrunken with condensed nuclei. Thus severe damage to the haemopoietic, reticuloendothelial and lymphoid system of spleen was evident.

The electron micrographs of spleen also revealed peroxidative changes in the ER of cells, ER fragmentation and severe mitochondrial damage with cell necrosis and autophagososmes inside the lymphoid cells. Thus severe damage to the cells constituting spleenic lymphoid tissue was evident. Since there was a lot of damage to the haemopoietic tissues of kidney and spleen, it was expected that these changes would reflect in the immune response of the fishes. The immunological study revealed a depression of cell mediated immunity and globulin concentration in the serum. There was also a decreasing trend for antibody response. Aflatoxin is

immunotoxic and causes extensive necrosis of lymphoblasts in mammals and birds (Thaxton and Hamilton, 1971; Paul *et al.*, 1977; Giambrone *et al.*, 1978; Pier,1986; Arkoosh and Kattari, 1987; Padmanabhan,1989; Rao *et al.*, 1990; Potchinsky and Bloom,1993). According to Roberts (1989) the spleen of the fishes is the organ where germinal centres and macrophages are abundant. The relative abundance of these cells and structures have been correlated with health status of fish (Wolke et al, 1985).

5.1.4.4. Thymus

In the thymus of aflatoxin treated fishes there were areas of haemorrhage and necrosis of lymphocytes. The ultrastructural studies also indicated severe destruction of thymic parenchyma. The significant depression of cell mediated immunity response evidenced by PHA test also supported the histological finding. Aflatoxin might have affected the T dependent lymhocytes and this may be the reason for the reduced response to PHA which is a T cell mitogen.

5.2. CADMIUM TOXICITY

In the present study, the LC50 for cadmium in *E. suratensis* was found to be 94 ppm. There are no previous reports of LC 50 value for cadmium specifically in *E. suratensis*. However, according to Moore and Ramamoorthy (1984), estuarine fishes have an LC 50 value for cadmium in the range of 8 –85 ppm. The present result appears to be near this range.

5.2.1. Haematology

The total erythrocyte count showed a significant fall in the cadmium-exposed fishes. This was contrary to what was seen in the case of aflatoxin treatment. However, there was sudden increase in the erythrocyte count in the third fortnight. This fluctuation in RBC count could not be explained. In *L. rohita, C. catla, Anguilla rostrata, C. puntatus, Perca fluviatilis* and *Pleuronectes flesus* the total RBC count decreased on exposure to cadmium (Sjobeck and Larsson, 1978; Sjobeck *et al.*, 1984; Gill and Epple, 1993; Mukherjee and Sinha, 1993; Bala *et al.*, 1994;

Vincent *et al*, 1996). Many authors are of the view that cadmium either increases the erythrocyte count or doesn't affect at all (Tort *et al.*, 1990; Morsy and Protasowicki, 1990; Saravanan and Natarajan, 1991; Allen, 1993; Aziz *et al.*, 1993). The comparison of graphs of erythrocyte counts in control and experimental group indicated fluctuations in the erythrocyte counts in both groups at different time intervals. In fishes erythrocyte count undergoes marked fluctuations (Schaperclaus, 1986) and there is wide variation in the normal range of erythrocyte count (Ellis *et al.*, 1989). Therefore, the decrease and increase in erythrocyte count seen in both groups cannot be attributed to cadmium treatment alone.

The leucocyte count in cadmium treated fishes showed fluctuations during the experimental period. In the first fortnight it was not significantly different from control. In the second fortnight, it significantly decreased followed by a significant increase in the third fortnight. In the last fortnight again it fell to a level similar to second fortnight. Studies have revelaed that sublethal cadmium toxicity increases leucocyte count (Sjobeck and Larsson, 1978; Sjobeck *et al.*, 1984; Saravanan and Natarajan, 1991; Aziz *et al.*, 1993; Gill and Epple, 1993; Sastry and Sachdeva, 1994) but there are reports that cadmium decreases the leucocyte count (Morsy and Protasowicki, 1990). Another pollutant, titanium dioxide is found to increase the leucocoyte count in *O. mossambicus* (Nair and Suryanarayanan, 2000). Many of these experiments were for a short duration of less than a week where as the present experiment was continued for eight weeks. In this period, there was an initial decrease followed by a rise and finally it again decreased. This may be because, the initial four week exposure adversely affected the production of leucocytes. So later there may be an attempt by the animal to compensate for the lack of leucocytes in the blood. This could not be maintained due to the continued action of cadmium. These results call for a detailed study on the dynamics of leucocyte concentrations in heavy metal toxicity.

In cadmium-exposed fishes, the erythrocyte sedimentation rate (ESR) showed an increase compared to the control in the first fortnight. This rate continued up to the third fortnight and in the fourth fortnight, there was a significant increase in the ESR. In the second and third fortnight, though the ESR increased to 10mm/2hr, it was not significantly different from control. Only in the fourth fortnight, there was

significant difference between the control and the treatment group. There is limited information on the ESR values of normal fish blood as well as in heavy metal toxicity. Hence it is very difficult to interpret the present results. In *C. carpio* and *A. testudineus*, exposure to high concentration of cadmium for 24 to 90 hrs caused an increase in the ESR values (Beena and Viswaranjan, 1987; Banerjee and Kumari, 1988). However, organic compounds and titanium dioxide are found to increase the ESR in *Heteropneustes fossilis* and *O. mossambicus*. The linear alkylbenzensulphonate causes pronounced increase in ESR at LC_{50} values (Nair and Suryanarayanan, 2000; Rani *et al.*, 2002). Okpokwasili and Ogbulie (1995) are of the opinion that, pollutants do not affect ESR. Verma and Panigrahi (1998) also have stated that lethal and sublethal concentrations of agrofen do not affect the ESR in *O. mossambicus*. Thakur and Bais (2000) have demonstrated that in *H. fossilis*, aldrin reduces the ESR while fenvalerate increases the ESR. Onusiriuka and Ufodike (2000) have shown that the ESR in *Clarias gariepinus* increases on exposure to toxic extracts of plants. Chlordane and Malathione did not affect the ESR in *Notopterus notopterus* (Gupta *et al.*, 1995). Atamanalap *et al* (2002) have noticed increases in the ESR of rainbow trouts due to the effect of cypermethrin. It is clear that there is no uniformity of ESR value changes in response to toxins. The ESR response varies depending on the species and the type of insult. The present results are probably the first information from *E. suratensis* about cadmium toxicity associated ESR changes. In the present study the high ESR values noticed in the fourth fortnight may be due to the effect of cadmium since the control group elicited a low ESR which was comparatively stable throughout the experimental period. A detailed study of normal ESR values of fishes is required for interpretation of these results.

The packed cell volume (PCV) in the cadmium-exposed group showed a decrease in the first, second and fourth fortnights where as the third fortnight registered a higher value. However, the general trend in the cadmium treated fishes was a decrease in the PCV. Many authors are of the view that cadmium significantly decrease the haematocrit values in fishes (Sjobeck and Larsson, 1978; Beena and Viswaranjan, 1987; Banerjee and Kumari, 1988; Gill and Epple, 1993; Vincent *et al*, 1996) where as an increase has been noticed by a few others (Morsy and Protasowicki, 1990; Aziz *et al.*, 1993). Changes in haematocrit in cadmium-exposed fish are influenced by several factors like concentration of the toxicant, time of

exposure and the fish species studied (Ruparelia *et al*, 1990; Gill and Epple, 1993; Palackova *et al.*, 1994). Therefore, the variation between the present study and previous ones can be attributed to the difference in exposure time as well as the fish species studied. However, the trend in PCV is in conformity with that of total erythrocyte count.

5.2.2.Serum factors

The total protein values did not show significant difference between the treatment and the control groups in the 2^{nd}, 6^{th}, and 8^{th} week while in the 4^{th} week a significant decrease was noticed. However, the trend in the treatment group was a general decline in the total protein as the duration of the cadmium exposure increased. This is very similar to the observations in *L. rohita,* and *C. punctatus* during cadmium toxicity (Mukherjee and Sinha, 1993; Sastry and Sachdeva, 1994). However, in tilapia, cadmium caused an increase in serum proteins. Suppression of protein synthesis in the cell is indicated by changes like increased heterochromatin and perichromatin granules in the nucleus and degranulation of endoplasmic reticulum (Porter and Bonneville, 1964; Scarpelli and Trump, 1964; Stenger, 1970; La Via and Hill, 1971; Trump *et al*, 1973; Thomson, 1984). In the present study, the electronmicrographs revealed ultrastructural changes like those mentioned above and therefore the decrease in total protein can be attributed to these ultrastructural changes.

The albumin and globulin values also registered decreased values in the cadmium-exposed fishes throughout the experimental period. Studies pertaining to the cadmium-induced effects on albumin/globulin in fish are scanty. In *C. carpio,* the serum albumin was lowered during cyanide toxicity (Kang and Jee, 1999). In crucian carp, a reduction in albumin was noticed due to the effect of sludge in rearing ponds (Chen *et al*, 1997). The reduction in albumin and globulin values observed in the present study clearly substantiates the decrease in total proteins.

The alkaline phosphatase concentration generally showed a decreasing trend in the treatment group though it returned to normal values at the end of the exposure period. In a number of fishes like *Tilapia nilotica*, *M. cephalus*, *H..fossilis*, *N.*

notopterus and *Fundulus heteroclitus*, there was a decreased activity or inhibition of alkaline phosphatase as a result of cadmium exposure (Jackim,1974; Hilmy *et al.*, 1985 Ghosh and Chakraborti,1991; El Demerdash and Elagamy,1999).The present result also agrees with the above findings. However, there are also contrary reports of increase in the enzyme activity during cadmium treatment (Shakoori *et al.*, 1990; Gill *et al.*, 1991; Reddy and Bhagyalakshmy, 1994). The liver endothelial cells and the biliary cells are a rich source of alkaline phosphatase (Teh and Hinton, 1993). The histological study of cadmium treated animals showed fatty degeneration of hepatocytes and general necrosis of the hepatic parenchyma, which included the biliary cells. This may account for the decrease in alkaline phosphatase. Later moderate hyperplasia of biliary epithelium was noticed. That may be the reason for increase in the alkaline phosphatase activity at later stages.

ALT is a cytoplasmic transaminase found in hepatocytes and this enzyme fluctuates in the serum of fishes in response to toxicity and stress. Necrosis of hepatocytes release a large amount of these enzymes to the serum and the estimation of these enzymes is a diagnostic tool to assess liver damage in human and veterinary medicine (Loc.cit). In cadmium treated fishes, initially ALT showed significant increase upto the 2^{nd} week. This was followed by a decrease in the fourth week. In the 6^{th} and 8^{th} weeks also it still remained decreased though not significantly different from the control. The initial increase can be attributed to the degeneration of smooth endoplasmic reticulum, which release the enzyme from the cytoplasmic matrix. This might have been followed by a decrease due to the non availability of the degenerating cells. In hepatic necrosis also high levels of serum enzymes will be available for hours only rather than for weeks. In *C mrigala, C carpio, C punctatus, Carassius auratus,* and *Scylla serrata*, ALT was significantly elevated following sublethal exposure to cadmium (Shakoori *et al.*, 1990; Zikic *et al.*, 1997, 2001; Sastry and Sachdeva, 1994; Reddy and Bhagyalakshmi, 1994) where as some authors have reported decreased ALT activity (Gill *et al.*, 1991). It is clear form these studies that the increase/ decrease of ALT in heavy metal exposed fishes depend on the time of collection of blood /serum. This work has shown that in the interpretation of serum ALT values, the duration of exposure of the metal has to be considered. If exposure time is more, there is a possibility of getting decreased values for the enzyme activity.

AST is a mitochondrial enzyme, which is released following mitochondrial damage. In the present study the AST values increased up to the 2nd week followed by a significant fall in the 4th week. The pattern of AST values is very similar to ALT. This again shows that the enzyme concentration values are entirely dependent on the time of estimation. An increase in AST values following cadmium treatment has been reported in various fishes like *C. mrigala, C. carpio, C. punctatus, C. auratus* and in the crab, *Scylla serrata* (Shakoori *et al.*, 1990; Zikic *et al.*, 1997, 2001; Sastry and Sachdeva, 1994). The present study also yielded similar results and the extensive mitochondrial damage to cadmium treated fishes accounted for the increase in the enzyme. However, this could not be sustained for a long time probably because of the extensive damage to the liver.

The response of ALT and AST to cadmium is different from that of aflatoxin wherein the two transaminases continued to increase throughout the experiment. This may be due to the difference between responses of the liver of fishes exposed to cadmium and aflatoxin. In aflatoxin treated fishes, in conjunction with hepatocyte degeneration and necrosis there was attempt for hepatocyte regeneration, which was indicated by the presence of hepatomegalocytes and basophilic foci. In cadmium treated fishes, there was extensive necrosis as exposure time increased. Hence, viable cells were not available in the later part of the exposure period. In case of aflatoxin treatment, newly formed cells might have again undergone degenerative changes in the later days. This had maintained the higher level of enzymes.

5.2.3. Vital organs

5.2.3.1. Liver

In cadmium treated fishes, there was general coagulative necrosis of hepatocytes and fatty change in the hepatocytes. As exposure time increased, the necrosis became more extensive and proliferation of fibrous tissue was noticed.

The liver is one of the target organs for cadmium toxicity (Giles, 1988). Many histological studies of the liver of fishes exposed to cadmium at different

concentrations are available even though ultrastructural studies are scanty. Usha Rani and Ramamurthy (1989) have noticed vacuolar degeneration of hepatocytes, fatty changes in the peripancreatic hepatocytes and necrosis of pancreatic cells in *T. mossambica.* Lemaire and Lemaire (1992) and Lemaire (1993) have reported perivascular fibrosis in the liver of *Anguilla anguilla* exposed to cadmium. The liver changes are irreversible (Lemaire, 1993). Degeneration of hepatocytes is a major lesion in the liver of *C. carpio* exposed to different concentrations of cadmium (Sovenyi and Szakolczai, 1993). Morsy and Protasowicki(1990) have also reported pathological alterations in the carp. Lipid peroxidation in the liver is a major lesion in *C. punctatus* and *Clarias batrachus* in cadmium toxicity (Rana and Singh, 1996). Cadmium and other metals have been found to stimulate lipid peroxidation in fish causing serious tissue damage, liver being the major target organ in which a marked fibrosis occurs (; Bano and Hansun, 1989; Lemaire and Lemaire, 1992; Thomas and Wofford, 1993). Necrosis of hepatocytes in fishes has also been observed as a result of pollution and other toxic insults (Bhattacharya *et al.*, 1985; Cruz and Tamse, 1986; Jambulingam, 1988).

The insult of cadmium to hepatocytes could be assessed in a better way by the ultrastructural studies. The nuclei of hepatocytes contained clumped or condensed chromatin along with large amount of chromatin and perichromatin granules indicating loss of DNA function in protein synthesis (Porter and Bonneville, 1964; Scarpelli and Trump, 1964; Stenger, 1970; La Via and Hill, 1971; Trump *et al.*, 1973; Thomson, 1984). Degranulation of the ER leading to the loss of ribosomes also indicated the cessation of protein synthesis. This was reflected in the serum protein values and enzyme changes in the serum. In fishes, stress due to heavy metal pollutants disrupt osmoregulation and specific physiological processes. In marine and brackish water fishes, the osmoregulatory processes involve excretion of sodium and chloride ions and retention of potassium ions. These are disturbed by the pollutants (Thaker *et al.*, 1996; Webb and Wood, 1998; Alkindi *et al.*, 1996). In the present study the ER appeared dilated which indicates the loss of ionic balance of the cell (Chevellie, 1983;Thomson, 1984). In later micrographs, progressive fragmentation of ER was seen. This is a peroxidative process seen in many toxicities (Zwefach *et al.*, 1965; La Via and Hill, 1971). In the present study also there are evidences of lipid peroxidation. The swelling of the polyribosomes had definitely affected the structural

protein synthesis and might have affected the structure and integrity of the cell. The mitochondrial damage observed establishes the adverse effects of cadmium on the energy generation and cell respiration. This is further evidenced by the loss of nuclear membrane integrity and detachment of cells from neighbouring cells, which clearly indicates the progression of cell death. Autophagic vacuoles were present in many cells. The presence of autophagy indicates cellular degeneration (Scarpelli and Trump, 1964; Zwefach *et al.*, 1965; Cheville, 1983). The findings of the present study establish severe histopathological damage to the liver and this is further confirmed by ultrastructural study. There are very few ultrastructural studies on cadmium toxicity in fish though such studies are abundant in mammalian toxicology. The present study is unique in this respect. It clearly shows that cadmium has got severe adverse effect on liver, which in turn affects the general metabolism and physiology of the fish.

5.2.3.2. Kidney

Kidneys of cadmium exposed fishes revealed severe damage to the tubular epithelial cell linings including vacoulation and necrosis. Extensive changes were noticed in glomeruli like accumulation of leucocytes around the Bowman's capsule, thickening and fibrosis of the Bowman's capsule, shrinkage of glomeruli and intercapillary thickening of glomeruli. In some glomeruli, increased nuclearity indicating proliferation of the cells was evident. Almost all glomeruli were affected in cadmium treated fish. The ultrastructural changes also revelaed severe insult to the kidneys. These changes were manifested by the fusion of microvilli of epithelial cells and in later stages the microvilli were totally lost. Degranulation and fragmentation of ER, mitochondrial swelling, loss of granules and cristae of the mitochondria and condensation of the matrix were also observed. These changes were noticed in the hepatocytes also. The reasons for these changes are already discussed in connection with hepatocellular alterations (Loc-cit). There was thickening of the membranes of the foot processes of the podocytes adhering to the epithelial cells. These thickened areas appeared electron dense and this finding supported the changes observed in glomerular capillaries. Such alterations are also noticed in glomerular nephritis in higher animals wherein deposits appear between endothelial basement membrane and cell membrane of the podocytes adhering to the basement membrane (Jones and Hunt, 1983). This is an important observation since, so far no

such reports are available in fishes. The increased nuclearity of the glomeruli may be due to the proliferation of mesenchymal cells. Increased proliferation of mesenchymal cells is noticed in higher vertebrates in response to glomerular injury (Cassey *et al.*, 1979; Slauson and Lewis, 1979; George and Somvanshi, 1979; Jones and Hunt, 1983; George and Seshadri, 1983). The changes like thickening, hyalinization and dilatation of glomerular capillaries, shrinkage of glomeruli and dilatation of Bowman's capsule are observed in experimental toxic studies with zinc, copper, cadmium, KMnO4 and beta hexachlorocyclohexane in a number of species of fishes (Kumar and Panth, 1981; Saxena, 1981; Cruz and Tames, 1986; Wester and Canton, 1986). The mesenchymal cell proliferation and sclerotic (fibrotic) changes in the glomeruli were noticed in this study. Such changes are very similar to those described for zinc toxicity by Dinesan (1988). The tubular epithelial cell changes noticed in the present study are very similar to the observations of Oronsaye (1989), Guo *et al.* (1989), Morsy and Protasowicki (1990), Sovenyi and Szakolczai (1993) and Rana and Singh (1996) in *C. carpio, Gasterosteus aculeatus, Hypophthalmycthis molitrix, C. punctatus,* and *C. batrachus*. The haemopoietic areas appeared empty. In ultrastructural studies, the cells exhibited severe degenerations. The nuclei appeared empty and cell architecture was lost. Cytoplasm appeared highly vacuolated and mitochondria severely condensed. All these changes indicated severe necrosis of haemopoietic tissue (Dinesan, 1988).

There is evidence for a relationship between exposure to environmental pollutants and kidney disease, but the complete data for fish are missing (Adham *et al*, 2002). The severe necrosis in haemopoietic tissues observed in the present study, though not reflected in the RBC count, resulted in a substantial reduction in the WBC count. Leucocytes are involved in the resistance against infectious diseases. The reduction in their number can adversely affect the health of the animal. Hence heavy metals in rearing water may indirectly affect the resistance of the fishes , which in turn makes them susceptible to various diseases.

5.2.3.3. Spleen

Spleen of cadmium treated fishes revealed loss of cells in the ellipsoids and depletion of lymphocytes from parenchyma. Many lymphocytes were observed to have shrunken pyknotic nuclei. These findings were substantiated by the electron micrographs where the lymphocytes showed extensive degenerative changes like condensation of the chromatin in the nucleus, mitochondrial damage and vacoulation of cytoplasm. The vacuoles contained electron dense materials.

Relative abundance of macrophages in spleenic ellipsoids, lymphoid tissue and germinal centers have been suggested as health indicators of wild fish (Wolke *et al.*, 1985). The variation in size and number of these spleenic parenchymal cells indicates ageing, starvation, toxicity and disease (Brown and George 1985; Wolke *et al.*, 1985). There is very little information on the effect of heavy metals on spleen. However, the present observations reveal loss of macrophages and lymphoid cells *in spleenic* tissues. Such changes can definitely affect the immune response of the fishes.

5.2.3.4.Thymus

The thymus in cadmium treated fishes showed extensive necrosis. The ultrastructural studies revealed vacant areas devoid of cells. Cells present were having damaged and condensed mitochondria and nucleus had clumps of heterochromatin. The cells appeared detached form the nursing cells. The architecture of the thymus was destroyed. The thymus structure in control fishes revealed large number of sinuses in which lymphoblasts were proliferating. There were large interdigitating epithelial type of cells. These cells had large tubular interdigitating cytoplasmic processes, which were in close contact with the lymphoblasts. Some cells contained electron dense granules. In cadmium treated fishes such a structure was completely absent. There are no earlier studies regarding cadmium toxicity on thymus of fishes. However, cadmium chloride was shown to decrease the cytotoxic leucocyte activity in tilapia (Smith *et al*, 1999). Thus, the present observations indicates the adverse effects of damage to the thymus on the immune response of the fish, especially the cell mediated immunity.

5.2.3.5. Gills

In the gills of the cadmium treated fishes, secondary lamellae appeared elongated. Some secondary lamellae were clubbed together and fused. Hyperplastic changes were seen at the base of the lamellae and the epithelial cells were desquamated.

The transition metals and heavy metals are known to insult gills (Lauren and Mc Donald, 1985; Verbost *et al.*, 1987). The gill is a sensitive organ and the pollutants cause acute ionoregulatory disruption in fish (Hinton, 1993). Hyperplasia of undifferentiated epithelial cells results in clumping and fusion of lamellae. It is a nonspecific lesion associated with a wide variety of insults including metals. Mucus cell hyperplasia has been reported in trout exposed to low pH (Daye and Garside, 1976).

At sublethal concentrations, toxicants have been shown to induce chloride cell hyperplasia. The changes noticed in the present study have been reported by a number of authors in cadmium toxicity (Versteeg and Giesy, 1986; Sunila, 1988; Gill *et al.*, 1988; Oronsaye, 1989; Morsy and Protasowicki, 1990; Lemaire, 1993; Sovenyi and Szakolczai, 1993) as well as in toxicity of other heavy metals (Skidmore and Tovell, 1972; Kumar and Pant, 1981; Sultan and Khan, 1981). Mallat (1985) has given a list of gill structural changes induced by toxicants. The author has concluded that the irritant induced alterations in gill morphology are nonspecific. Therefore confounding etiologies result in similar pathology and the interpretation becomes more complicated. The proliferation of chloride cells is a compensatory and adaptive response to ion loss in fishes (Hinton, 1993). The hyperplastic changes observed in the present study might be an attempt by the animal to compensate for the stress due to cadmium and this explains the absence of apparent behavioural changes during the experimental period.

5.3. Conclusion

The present study has generated considerable amount of information regarding the toxic effects of aflatoxin and cadmium on *E suratensis*.

Among the various physiological indices studied, the impact of aflatoxin was pronounced in the case of total erythrocyte count, total leucocyte count, serum proteins and the enzymes alkaline phosphatase, alanine amino transaminase and aspartate amino transaminase. All the vital organs studied, viz, liver, kidney, spleen and thymus were severely affected as evidenced from the histological and ultrastructural alterations. A significant outcome of the study is the demonstration of preneoplastic lesions in the livers of aflatoxin fed fishes. This finding about the sensitivity of *E suratensis* to aflatoxin-induced hepatocarcinogenesis reveals scope for using this species in research on cancer, which at present is mostly concentrated on rainbow trout owing to its extreme sensitivity than any other fish.

Cadmium has also elicited remarkable toxic impacts on the physiology as well as the vital organs of the fish. Among the haematological and biochemical parameters investigated, alterations, which could be correlated to cadmium toxicity, were exhibited by total leucocyte count, serum proteins and the enzymes alkaline phosphatase, alanine amino transaminase and aspartate amino transaminase. Cadmium also caused severe damages to the vital organs like liver,kidney, spleen, thymus and gills of the fish. This could be clearly demonstrated in the histological and ultrastructural studies. Thus the study has brought to light the precarious nature of the sublethal effects of the pollutants on the fish. These damages caused at the subcellular levels, if left unchecked, can spread to the higher levels of biological organization viz, organ, organ system, organism, population, communities and ultimately the ecosystem as a whole.

Summary

SUMMARY

The routes of exogenous toxic insults to the aquatic organisms can be broadly grouped into two viz, food and the environment. The pathological alterations in *E. suratensis* due to the effect of toxicants introduced through these two routes were studied using aflatoxin and cadmium wherein aflatoxin served as the food contaminant and cadmium as the environmental pollutant. *E suratensis,* is a euryhaline Asian cichlid with proven aquaculture potential and is widely distributed in the inland water bodies of peninsular India. Hence it is an ideal species for studies relating to nutritional pathology as well as ecotoxicology.

In the present study, two sets of experiments were conducted in *E suratensis* viz, on aflatoxicosis and cadmium toxicity.

In the experiment on aflatoxicosis, the fishes were reared for eight weeks on two diets namely treatment diet (aflatoxin incorporated feed @ 400ppb) and normal feed (control diet). Fortnightly samples of blood and vital organs were taken from both the groups to assess the physiological alterations as well as insults to the vital organs. The results are briefly summarized as follows:

- Remarkable behavioural changes were not observed between the treatment and the control groups.

- The gross pathology of the internal organs on termination of the experiment revealed numerous black spots and raised white patches on the liver of aflatoxin treated fishes.

- The total erythrocyte count registered an initial decrease followed by an increase in the treatment group when compared to the control.

- The total leucocyte count in the treatment group showed a general increase when compared to that of control group.

- The Erythrocyte Sedimentation Rate (ESR) elicited a confounding a response. In the treatment group it was very high initially as well as at the end of the experiment. However this could not be correlated with the serum protein concentrations on which the ESR is highly dependent.

- The Packed Cell Volume initially did not exhibit much variation between control and treatment groups, but it was significantly high in the treatment group after 6^{th} week of the experiment.

- The serum factors like total protein, albumin and globulin showed a general decrease in the treatment group compared to control.

- The serum alkaline phosphahtase, alanine aminotransaminase and aspartate aminotransaminase registered an increase in the aflatoxin fed fishes.

- The immunological indices like sensitivity to PHA and antibody titre against *A. hydrophila* were depressed in aflatoxin treated fishes.

- The livers of the aflatoxin fed fishes exhibited progressive damage as evidenced in the histological and ultrastructural studies. Initially the hepatocytes showed vacoulation and the stromal connective tissue became prominent. Subsequently, focal coagulative necrosis followed by fibroblastic and biliary proliferation was noticed. Infiltration of mononuclear leucocytes around proliferating biliary ducts was also noticed. The livers with black spots on surface revealed areas of hepatic cell regeneration as evidenced by cells with basophilic cytoplasm and vesicular nuclei. Basophilic foci and megalocytosis were also noticed. In three cases, the liver parenchyma was invaded by pleomorhic, polyhedral basophilic cells having hyperchromatic nuclei and high mitotic index, which were similar to the cells of

hepatocarcinoma and the lesions were diagnosed as hepatocellular carcinoma.

- The ultrastructural changes in the hepatocytes included vacuolation, appearance of liposomes in the cytoplasm, degranulation of rough endoplasmic reticulum (RER), proliferation of endoplasmic reticulum, mitochondrial damage, autophagia, presence of perichromatin and chromatin granules and protein inclusions in the nucleus, appearance of multivesicular bodies with condensed organelles and damaged desmosomes.

- The kidneys of the aflatoxin exposed fishes showed severe histomorphological alterations. The epithelial cells of proximal convoluted tubules lost its brush border appearance. The cells became shrunken and desquamated and some had vacuoles inside the cytoplasm and many of them desquamated into the lumen. The glomerular capillaries showed focal thickening and glomeruli developed adhesions with the parietal layer of Bowman's capsule. The basement membrane of Bowman's capsule became thickened and the parietal epithelial layer proliferated. Fibrosis of Bowman's capsule was also noticed. The haemopoietic tissue also showed degenerative changes.

- The ultrastructural studies revealed changes in RER, mitochondria, nuclei and lysosomes. The ER fragmentation, loss of ribosomes and various mitochondrial changes seen in hepatocytes were also seen in epithelial cells. The nuclei showed heterochromatin formation and conformational changes in the nuclear membrane

- In the spleen of aflatoxin treated fishes, the ellipsoids were depleted of macrophages. The spleenic white pulp also contained fewer lymphocytes and the lymphocytes present were shrunken with condensed nuclei. The electron micrographs of spleen also revealed peroxidative changes in the ER of cells, ER fragmentation, severe

mitochondrial damage with cell necrosis and autophagosomes inside the lymphoid cells.

- In the thymus of aflatoxin treated fishes there were areas of haemorrhage and necrosis of lymhocytes. The ultrastructural studies also indicated severe destruction of thymic parenchyma.

In the study on cadmium toxicity, LC_{50} of cadmium for *E. suratensis* was found out and one tenth of this was used for conducting the chronic toxicity study. In the chronic toxicity study of eight weeks, two groups of fishes were maintained in which one group was exposed to cadmium while the other group served as the control. Fortnightly samples of blood and vital organs were taken from both the groups to assess the physiological alterations as well as insults to the vital organs. The results are briefly summarized as follows:

- LC_{50} of cadmium for *E. suratensis* was found to be 94 ppm.

- No marked behavioural changes were observed due to cadmium toxicity.

- The total erythrocyte count showed a significant fall in the cadmium-exposed fishes during the first two fortnights. However, there was a sudden increase in the erythrocyte count in the third fortnight.

- The leucocyte count in cadmium treated fishes showed fluctuations during the experimental period. In the first fortnight it was not significantly different from the control. In the second fortnight, it significantly decreased followed by a significant increase in the third fortnight. In the last fortnight again it fell to a level similar to second fortnight.

- The erythrocyte sedimentation rate (ESR) was significantly high in the treatment than the control in the first and fourth fortnights. On 2nd and 3rd fortnights, ESR did not vary significantly between the treatment and the control groups.

- The packed cell volume (PCV) in the cadmium-exposed group showed a decrease in the first, second and fourth fortnight where as the third fortnight registered a higher value. However, the general trend in the cadmium treated fishes was a decrease in the PCV.

- The total protein values did not show difference between the treatment and control group in the 2nd, 6th and 8th week while in the 4th week a significant decrease was noticed. However, the trend in the treatment group was a general decline in the total protein as the duration of the cadmium exposure increased.

- The albumin and globulin values also registered decreased values in the cadmium-exposed fishes throughout the experimental period.

- The alkaline phosphatase activity generally showed a decreasing trend in the treatment group though it returned to normal values at the end of the exposure period.

- In cadmium treated fishes, the serum alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) elicited a similar response. Both the enzyme activities registered an increase initially in 2nd week, but decreased from the 4th week onwards.

- In cadmium treated fishes there was a general coagulative necrosis of hepatocytes and fatty changes in the hepatocytes. As exposure time increased, the necrosis became more extensive and proliferation of fibrous tissue was noticed.

- The ultrastructural studies of hepatocytes from cadmium exposed fishes revealed clumped or condensed chromatin along with large amount of chromatin and perichromatin granules. Degranulation, fragmentation and dilatation of the ER, swelling of the polyribosomes, mitochondrial damage, loss of nuclear membrane integrity and detachment of cells from neighbouring cells were the other observations.

- Kidneys of cadmium exposed fishes revealed severe damage to the tubular epithelial cell linings, which includes vacoulation and necrosis. In glomeruli, accumulation of leucocytes around the Bowman's capsule, thickening and fibrosis of the Bowman's capsule, shrinkage of glomeruli and intercapillary thickening of glomeruli were observed. In some glomeruli increased nuclearity indicating proliferation of the cell was evident.

- The cadmium induced ultrastructural changes in kidneys included fusion of microvilli of epithelial cells and in later stages the microvilli were lost. Degranulation and fragmentation of ER, mitochondrial swelling, loss of granule and cristae of the mitochondria and condensation of the matrix were also observed. There was thickening of the membranes of the foot processes of the podocytes adhering to the epithelial cells and these thickened areas appeared electron dense.

- Spleen of cadmium treated fishes revealed loss of cells in the ellipsoids and depletion of lymphocytes from parenchyma. Many lymphocytes had shrunken pyknotic nuclei. In the electronmicrographs the lymphocytes showed extensive degenerative changes like condensation of the chromatin in the nucleus, mitochondrial damage and vacoulation of cytoplasm. The vacuoles contained electron dense materials.

- The thymus in cadmium treated fishes showed extensive necrosis. The ultrastructural studies revealed vacant areas devoid of cells. Cells

present had damaged and condensed mitochondria and nucleus had clumps of heterochromatin. The cells appeared detached form the nursing cells. The architecture of the thymus was destroyed.

- In the gills of cadmium treated fishes, the secondary lamellae appeared elongated and some secondary lamellae were clubbed together and fused. Hyperplastic changes were seen at the base of the lamellae and the epithelial cells were desquamated.

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ANNEXURE-I

ALCOHOL GRADIENT USED IN AUTOMATIC TISSUE PROCESSOR FOR HSITOPATHOLOGY

1. 50%	-2hrs
2. 70%	-2hrs
3. 90%	- 2hrs
4.Absolute alcohol	- 1hr
5. Propanol-I	- 1hr
6.Propanol-II	- 1hr
7. Propanol-III	- 1hr

ANNEXURE-II

ACETONE GRADIENT USED IN TRANSMISSION ELECTRON MICROSCOPY

1.30%	- Two changes of 20 min each
2. 50%	- Two changes of 20 min each
3. 70%	- Two changes of 20 min each
4. 80%	- Two changes of 20 min each
5. 85%	- Two changes of 20 min each
6. 90%	- Two changes of 20 min each
7.95%	- Two changes of 20 min each
8. Absolute acetone	- Three changes of 30 min each